THESIS

MORPHOLOGY AND ACTIVITY OF GONAD IN MEDIAN-STRIPED BURROWING FROG, *Kaloula mediolineata*, IN DIFFERENT SEASONS

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DISSERTATION

MORPHOLOGY AND ACTIVITY OF GONAD IN MEDIAN-STRIPED BURROWING FROG, *Kaloula mediolineata*, IN DIFFERENT SEASONS

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Gonadal morphology and seasonal differences of reproductive gonadal cycle in free-living population of median-striped burrowing frog, *Kaloula mediolineata* were examined. Light and transmission electron microscopy were used to study gonads in this frog species. Gonad samples were collected throughout one year period. Sectioned testes were assessed for spermatogenetic activity. The mean diameter of seminiferous tubules and Leydig cells nucleus per individual were obtained. Based on the nuclear characteristics, twelve stages of male germ cells in the seminiferous tubules can be classified. Developing oocytes in adult females can be divided into six stages based on size, color, histological and ultrastructural observations.

K. mediolineata belongs to the continuous type of spermatogenetic cycle. Males bearing spermatozoa were present practically year-round and spermatogenetic activity showed a regenerative phase from the cold season to the end of the wet season, and a brief degenerative phase in October. These characteristics resemble those from species with continuous reproductive cycle. Recruitment of previtellogenic oocytes to vitellogenic growth commences in the hot season (March) through the wet season. The presence of previtellogenic oocytes during May to August was correlated with the large amount of spermatozoa in the testes in male frogs. However, the amplexing occurred only in two months of high rainfall (May and August) associated with high soil moisture and surplus water form the temporary pond. Distinct seasonal differences in morphological appearance of Leydig and Sertoli cells were apparent when observed at different periods of reproductive gonadal cycle.

Student's signature

Thesis Advisor's signature

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Surapol Ardsoongnoen

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INTRODUCTION

The major threats to the continued survival of the biodiversity of Thai amphibians are habitat destruction, uncontrolled utilization, and lack of pertinent knowledge on biology and ecology of most indigenous species. In light of the biology of these amphibians, the understanding of their reproductive biology seems vital for its survival. As far as studies examining of the reproductive biology in amphibian species inhabiting Thailand is very limited.

Amphibians are sensitive indicators of environmental change (Blaustein *et al.*, 1994; Duellman and Trueb, 1994). Their skin is not protected by hair or feathers; their eggs lack hard outer shells, these exploring them to environment. Currently, there is intense interest worldwide in the effects of many environmentally important chemicals on the reproductive biology of amphibians, particularly environmental estrogens. By disrupting morphology of gonad and developmental processes, they may impair not only the individuals exposed but also have lasting influence on their offspring as well. Environmental estrogens include many herbicides, fungicides, insecticides, and industrial chemicals. Environmental estrogens disrupt reproductive endocrine function by binding primary to estrogen receptors. These estrogenic contaminants are responsible for the decline in reproductive success and led to study morphological characters of the reproductive system (Palmer and Palmer, 1995). In Thailand, heavy chemical use in an agricultural activity has made Thai amphibians less abundant (Graham and Round, 1994). Thus there is crucial need for more research on Thai amphibians to gain our knowledge of their biology and improve their conservation status. For Thailand, in times of hardship some Thai villagers in poor regions depend partly on frogs because they are readily available and provide good source of protein supplement

Among Thai amphibians, the median-striped burrowing frog, *Kaloula mediolineata*, is chosen for the present study because it is listed as the endemic species of Thailand. In addition, information regarding the life history of this frog species is greatly to be desired (Taylor, 1962). So, its reproductive biology should be studied. Moreover, the amount of

median-striped burrowing frog consumed locally is obtained by increasing of capture from nature, especially northeast villagers, for a long time. At present, population of this frog species declines rapidly and disappears from some local range every year. This is due to overexploitation by capturing for consumption.

In the present study, therefore, it starts describing such biology systematically in free living population of the median-striped burrowing frog. Initially the focus of this systematic will be clarified on the morphology of the gonad as well as the gonadal cycle. In this sense, histology and ultrastructural features are powerful tools which may give specific and complementary data. Moreover, the result in the present study may expand knowledge to further study on its reproductive ecology by means of biotechnology. In addition, information concerning its gonad can be used as the morphological assay of environment effects on population of *K. mediolineata*. Finally, the knowledge gained is a prerequisite of effective conservation programmes regarding this endemic species and therefore sustaining our rich biodiversity.

Objectives

The objectives of this study are as follows

1. To study the testis structure and the classification of germ cell by light and transmission electron microscopy.
2. To study the morphology and the classification of oocyte by light and transmission electron microscopy.
3. To study the morphology of testis and ovary in different seasons.
4. To elucidate the reproductive gonadal cycle and gonadal pattern.

LITERATURE REVIEW

The Frog

Kaloula mediolineata is listed as the endemic anuran species of Thailand's biodiversity (Office of Environmental Policy and Planing,1995). It occurs only in Thailand except for extreme south (Taylor, 1962 and Frost, 1985). This speies is rather large burrowing frog (Fig.3) which body length is about 56 mm. The distinct characteristics are elevated head in occipital region, lower short rounded snout, and pointed toes. It has dark above with broad light-yellow or light brownish marks from upper eyelid to groin and similar median stripe from middle of back to near vent. Its entire ventral side is yellowish white, however, male has blackish brown chin (Taylor, 1962)(Fig. 4). There are some reports regarding tadpole characteristics of this species (Heyer,1971 and Inthara, 2000). Taylor (1962) proposed that information regarding the life history of this species is greatly to be desired. *K. mediolineata* are generaly not seen above ground untill there is heavy rain. They are to be found in or near the same pools with *K. puchra*, *Gylphoglossus molossus* and *Calluella guttulata* to deposit eggs after heavy rain in breeding season (Taylor, 1962 and Heyer, 1971).

The Gonads

One reliable indicator of sexual phenotype in an individual is the type of gametes that it is capable of producing. The male gender is identified on the basis of its capcity to produce sperm, or spermatozoa, by the process of spermatogenesis. The female gender produces ova through oogenesis. Both types of gametes are produced by specialized structures called gonads. In males, functional gonads are referred as the testes while in females they are called the ovaries (Lombardi, 1998).

The Male Gonad

In most taxa, the testes are paired and situated along the dorsal wall of the coelomic cavity. Testes are either temporarily or permanently situated outside the coelomic proper within modified outpocketing of the coelomic space inside the scrotum. Within the testis, spermatogenesis, or production of sperm, takes place within the epithelial lining of pockets

or tubules known as ampullae or seminiferous tubules. Sometimes ampullae are enclosed to form follicle-like structures known as cysts, or spermatocysts (Saidapur and Shanbhag, 1999). Both structures consist of epithelial layer comprising of both somatic cells and spermatogonial cells. Somatic cells give rise to sustentacular cells, or Sertoli cells, that support and nourish sperm precursors during spermatogenesis. Ampullae or seminiferous tubules are bounded by outer basal lamina and elements of the testicular interstitial connective tissues. In addition to containing fibroblasts, blood vessels, and nerves, these conective tissues often contain specialized endocrine cells called interstitial cells, or Leydig cellls that are involved in androgen production (Lombardi, 1998; Pudney, 1993). Leydig cells and the spermatogenetic activities may or may not run parallel depending upon the animal species and its geographical location (Saidapur, 1989).

In most anurans, the testes are simple and ovoid structure lying ventral to the kidneys near their anteromedial border (Fig. 6). In several speceis of urodele amphibians, however, the testes are composed of distinct lobes arranged in linear chain and linked together with slender cords of tissues (Pudney, 1993; Saidapur and Shanbhag, 1999). It is suspended from the kidney within the body cavity by the portion of peritoneum designated as the mesorchium which one side is continuous with the dorsal peritoneum and the other side with the portion of peritoneum which covers most of the ventral surface of the kidney. The vas deferens of the testis lie between the testis and the ureter of the kidney. The anterior two-fifths of the kidney is often referred as the genital portion because of its close association with the gonad in contrast to the posterior three-fifth which is regarded as purely excretory part. Covering the anterior margin of testis and occasionally also part of the posterior, there are finger-like yellowish bodies which are the accumulation of fat or corpora adipora serving as the stage of reserved nutritive materials (Lofts, 1984).

The testes are surrounded by an elastic fibrous coat. They consist of convoluted seminiferous tubules lined by permanent germinal epithelium whose composition may vary seasonally or in accordance with the reproductive phase. The structurally simple testis of anurans increases in size and weight during spermatogenesis (Duellman and Trueb, 1994). As in other anamniotes, spermatogenesis is of the cystic type. Germ cell proliferation occures in coordinated clusters which each cluster is being enclosed within membranous capsule for much of its development. The capsule including its contained germ cells is termed as germinal cyst or nest or follicle. The cells present in a cyst are the same stage of

development and are derived from single spermatogonium. Each primary spermatogonium is completely enclosed by follicle cells which later mature into secretory Sertoli cells. During the progression of spermatogenesis these cells hypertrophy possess ultrastructure features characteristic of steroidogenesis cells, i.e, acquire smooth endoplasmic reticulum, spherical mitochondria with tubular cristae, lipid dropletes (Lofts, 1984; Saidapur, 1989). In cross section of seminiferous tubule, cell nests in different stages of spermatogenesis are encountered. Also, the sectioned testes display remarkable uniformity in gametogenetic composition throughout the whole gonad (Saidapur and Shanbhag, 1999).

Spermatogenic Patterns

Spermatogenesis is basically similar in all vertebrate classes, its cycle is completed in the testis (Duellman and Trueb, 1994). It starts with mitotic divisions of spermatogonia and progresses through the meiotic spermatocyte stages to the maturation stages as the spermatogenic wave, terminating in the insertion of spermatozoan bundles into the Sertoli cells (Jorgensen, 1992). In temperate anurans the sperm cells mature uniformly throughout the testis (Lofts, 1984), but in tropical species that breed throughout the year, the testis contains sperm cells in various stages of maturation. For example, in *Rana erythraea* in Borneo, each tubule contained sperm cells in only one stage of maturation but locules in one testis often contained cells in all stages of spermatogenesis (Inger and Greenberg, 1963). Annual spermatogenic cycles in anurans have been grouped into 3 categories: discontinuous, potentially continuous, and continuous types (Lofts 1974). The discontinuous type is commonly found in temperate-zone species such as *Rana esculenta* (Lofts, 1964) and *R. temporaria* (Lofts *et al.*, 1972). Generally it has discrete seasonal cycles of reproduction with pronounced changes in gonad size, gamete production, and sex accessory structure. The potentially continuous type exhibits partial cessation of spermatogenic activity during some seasons of the year but primary spermatogonia remain sensitive to gonadotropic stimulation. The continuous spermatogenesis is presumably characteristics of anuran and perhaps other amphibian males living in constant environments, for example, in the tropics, but analyses of spermatogenesis in male frogs living in such regions are lacking (Jorgensen, 1992). However, in tropical southern India where the temperature is constantly high, ranging between about 20^{o}C and 30^{o}C, the spermatogenesis status throughout the year has been quantitatively evaluated in five species in number of spermatozoan bundles and free sperm numbers within seminiferous tubules. *Bufo melanostictus*, *Rana cyanophlyctis* and *R.*

hexadactyla show little annual variation (Mondal and Basu, 1960; Thyagaraja and Sarkar, 1971; Saidapur and Nadkarni, 1975; Kanamadi *et al.*, 1983; Saidapur, 1989). One species, *R. tigrina*, however, show pronounced annual cyclicity in spermatogenesis which discontinue during winter and early spring (Basu and Mondal, 1961; Saidapur and Kanamadi, 1982). The last species, *B. marinus*, also shows annual cyclicity in spermatogenesis (Saidapur, 1989). Jorgensen (1992) reported both the species with continuous and with cyclic spermatogenesis could be collecteded in the same locality.

Classification of Testicular Cells

The classification of cells in the developing testis of anurans has been investigated by many researchers and *Xenopus laevis* is the choice of anuran for most experiments. In light microscope investigations the germ cells in testis had been classified into eleven stages based on premeiotic DNA synthesis appearances and size. These stages were primary spermatogonium, secondary spermatogonium, leptotene spermatocyte, zygotene spermatocyte, pachytene spermatocyte, diplotene spermatocyte, secondary spermatocyte, early spermatid, middle or round spermatid, late spermatid and spermatozoa. In addition, autoradiographic studies with tritiated thymidine were employed to determine the duration of spermatogenesis in *X. laevis*. Results showed duration of various cell types, the longest was twelve days in pachytene stages and the shortest was one day for diplotene stage (Kalt, 1976). Similarly in *Rana esculenta*, the longest duration was twelve day in pachytene stage with and the shortest duration was one day in diplotene and secondary spermatocyte stages. The duration of spermatogenesis from primary spermatogonium to the formation of spermatozoa was 41 days (Rastogi *et al.*, 1983). In the toad, *Bufo arenarum*, the germ cells were classified using both light and transmission electron microscopes into eight stages,they were primary spermatogonium, secondary spermatogonium, primary spermatocyte, secondary spermatocyte, early spermatid, round spermatid, late spermatid, and spermatozoa (Burgos and Fawecett, 1956). In comparison to other species of anurans, spermatogenesis and the classification of germ cells in the testis in *K. mediolineata* are still lacking. Therefore one primary purpose of the present study is to classify stages of the germ cells in the frogs' testes based on their characteristic morphology and to elucidate its spermatogenic pattern.

The Female Gonad

Depending on the taxon, ovaries are lamellar or hollow or solid. Lamellar ovaries occur in some teleosts where they consist of folds of the coelomic lining containing oocytes which are ovulated into coelom. Hollow ovaries are characterized by most vertebrates and, in most instances, develop through folding of the coelomic lining over tissues of the germinal ridge. Also cavity may develop through delamination of tissues at the center of the organ. In amphibians, the ovary is also hollow which the wall thrown up into series of folds. The wall consists of relatively narrow cortical region covering by germinal epithelium and being lining internally by epithelium derived from primodial medullarly tissues. Population of oocyte follicles in different stages of development embed in the connective tissues stroma (Lofts, 1984; Lombardi, 1998).

Female reproductive system of the frog consists of two major organs, the ovaries and oviducts (Fig. 7). In most anurans, the ovaries are characterized by multilobed organs. Ovaries lie to both sides of the vertebral column; there are 7-12 lobes on each side. The ovaries occupy most of the coelomic cavity. In the rainy season, the ovary is filled with large number of large and mature oocytes. The oocytes are forced out of the ovary to the coelomic cavity and, then, they are carried to the oviduct (Katagiri, 1987).

Morphology of Oocyte and its Classification

Basically, oogenesis is the same in all three groups of amphibians. The developing oocyte lies in the follicles associated with the ovary. Each developing oocyte in the frog's ovary is surrounded by layer of follicular cells which is enclosed by vascular network and epithelial membrane. Beneath the follicular cells is a thin vitelline membrane covering the surface of mature oocyte. The most striking characteristics of the anuran oocytes which differ from other classes of animals are the deposition of pigments as they exhibit dark color during mid-oogenesis and make distinct between the dark animal pole and the white vegetal pole during the last stage oocyte. The movement of germinal vesicle toward the animal pole also occurs during this time (Duellman, 1986).

Classification of developing oocytes of anurans was investigated by many reseachers (Grant, 1953; Kemp, 1953; Wartenberg, 1962; Balinsky and Davis, 1963; Dumont,

1972). *Xenopus laevis* is one of the most popular anurans for previous laboratory experiments. The oocytes were classified into six stages based on external appearance, color, and size (Dumont, 1972). Correspondingly in *Rana pipiens*, the same criteria was used to classify developing oocytes (Kemp, 1953). In addition, the classification of the developing oocytes is divided into four stages by mean of the up take of vitellogenin as follows: pre-vitellogenic, vitellogenic, post-vitellogenic, and mature follicle (Wittek, 1952). Previtellogenic oocytes increase in size nearly ten fold; nutrients for the growth are provided by the ovary via plasma membrane which forming as follicular stalk (Wallace *et al.*, 1970). Unfortunately, these morphological data and the classification of oocytes in *Kaloula mediolineata* are still lacking. Thus the purpose of the present study is to examine the morphology as well as the classification of the oocytes in this frog species.

Ovarian Cycle

Two categories of oocytes are evident in the amphibian ovary, small with gonadotropin-independent oocytes and larger with gonadotropin-dependent oocytes. Oogenesis proceeds synchronously in the two ovaries indicating that the initiation of oogenic episode is basically under systemic control but the factors that control oogenesis are still not well understood. Presumably, the mechanisms that control oogenesis and pool of small oocytes are of somatic-type growth control (Jorgensen *et al.*, 1979). The pool of small oocytes serves as reserve from which oocytes can be recruited to vitellogenic growth not only at sexual maturation and after ovulation but also after atresia of complement of vitellogenic oocytes (Vijayakumar *et al.*, 1971). The recruitment of complement of small oocytes to vitellogenic growth initiates an ovarian cycle which proceeds at uniform rate until the oocytes are full-grown. The pattern of vitellogenic growth can be assessed from the size and frequency of distributions of oocytes during different phases of the growth period. (Jorgensen 1973, 1975; Jorgensen *et al.*, 1979; Jorgensen, 1984). Vitellogenesis is a crucial period in the female reproductive cycle and is characterized by pronounced growth of oocytes. Much of these growth results from the uptake of specific proteins, predominantly hepatically-derived yolk protein precursor or vitellogenin or lipo-phosphoprotein (Wallace, 1985). All basic proteins are stopped during serum lipo-phosphoprotein synthesis (Follett, 1967). Vitellogenin is, then, transported to the ovaries where it is taken up by pinocytotic activity. After entering the cytoplasm, vitellogenin is sequesterated into two main components, phosvitin and lipovitelline (Wallace and Dumont, 1968). This may due to

proteolytic activity of lysosomes (Kaplan, 1981). Finally, these two components are reassociated and arranged in hexagonal yolk platelets.

Reproductive Pattern

Reproductive pattern seems to be correlated with the climatic condition prevailing in the habitats. Among anurans, two basic reproductive patterns are evident. Most tropical and subtropical species are capable of reproduction throughout the year in which rainfall seems to be the primary extrinsic factor in controlling the timing of reproductive activity. In most temperate species, reproductive activity is cyclic and dependent on a combination of temperature and rainfall (Jorgensen, 1992; Duellman and Trueb, 1994). In equatorial habitats with constantly warm and humid climate, amphibian may reproduce throughout the year, such as *Rana erythraea* in Borneo (Inger and Greenberg, 1963), and the toad, *Bufo melanostictus,* in Singapore and Djakata (Church, 1960; Bery, 1964). Gopalakrishnan and Rajasekharsetty (1977) studied the spermatogenetic cycle of *Rana hexadactyla* from two regions in India (Mysore and Mangalore) which vary in their climatological conditions and inferred that gonadal activity in *R. hexadactyla* is specifically under influence of the range of temperature and relative humidity and also with the pattern of rainfall in particular locality. In the region with pronounced wet and dry season, in southern India for example, the main breeding season of *B. melanostictus* coincides with monsoon rain, but spawning may occur outside this period (Jorgensen *et al.*, 1986).

MATERIALS AND METHODS

Materials

Median-Striped Burrowing Frogs were collected each calendar month between November,1999 and October, 2000 in the Nongteng-Chakkarat National Reserved Forest by excavating them from their burrows in the forest floor or capturing by hand on rainy night.

Methods

Collecting Area and Climate

The collecting area was located in the dry dipterocarp forest,Nongteng-Chakkarat National Reserved Forest, Chalerm Prakriat District, Nakorn Rachsima Province, which the Royal Development project was taken place since 1983. This area is approximately 30 km from Nakorn Ratchasima Province (Fig. 1). This national reserved forest covers an area approximately 81,875 rai on elevation range from 200 upto 250 m a.s.l. with inclination of 3-8%. Trees in this forest were cut for railway sleeper and fuel consumption during the last two decades. Remaining vegetations are only saplings and seedlings.

The area is influenced mainly by two monsoons, the northeast and the southwest monsoons. The period of wet season is from May to October and the period of dry season is from November to April. During 1969-1998, the average annual rainfall, relative humidity, and temperature were 1,047.6 mm, 74%, and 27.0°C respectively. The maximum temperature was 29.6°C and the minimum was 22.9°C (Wachrinrat, 2000). Thus, the climatological data from local meteorological station were collected in order to analyse relationship between the amount of rainfall and the gonadal cycle. The whole year is divided into three periods. The first period included the cold months from November to February which is the period of dry and relatively cold part of the year and coinciding with the north-east monsoon. The second period includs the months of March and April which is the hot season. The third period includes May to October which is the wet season (Fig. 2).

Figure 1 Map showing of the location of collecting area (●) : Nongteng-Chakkarat National Reserved Forest.

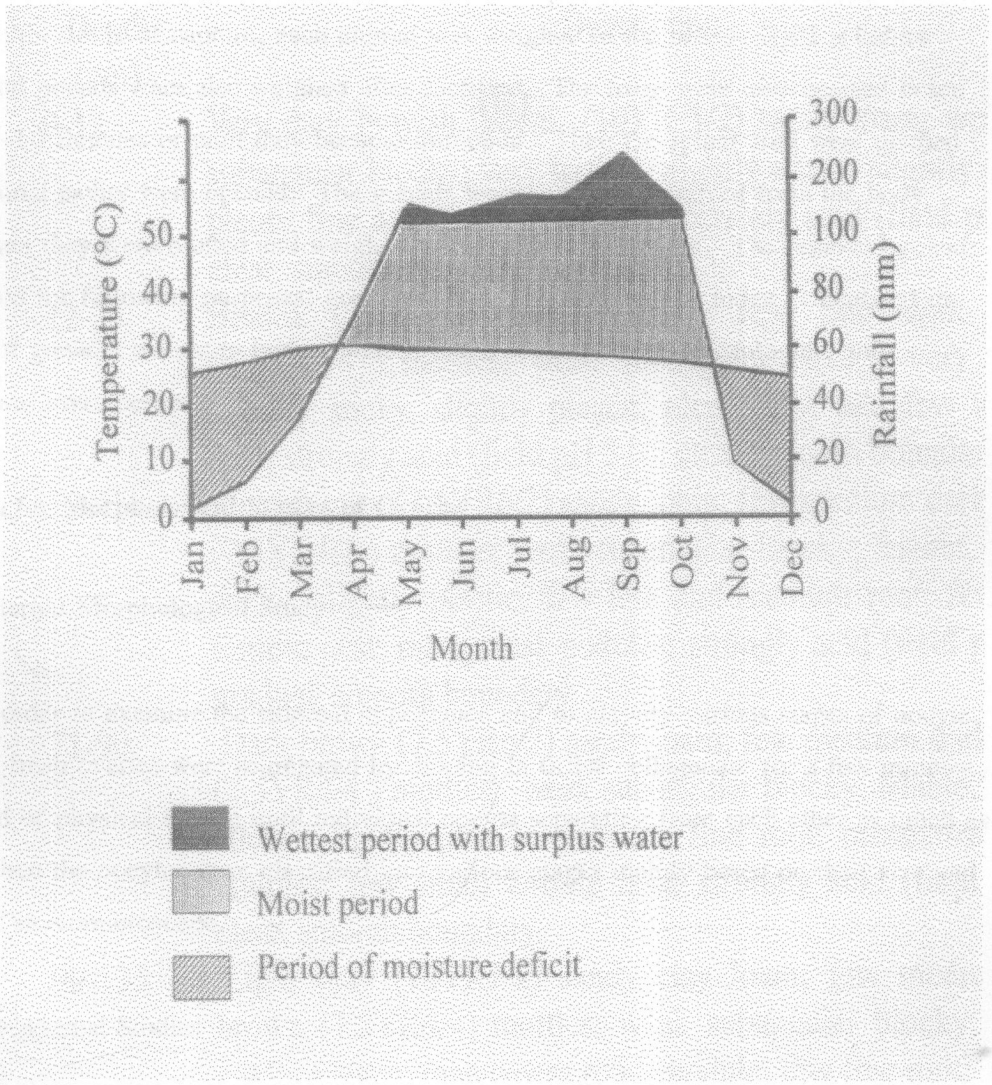


Figure 2 Walter's climatic diagram of Nongteng-Chakkarat National Reserved Forest, Nakhon Ratchasima (1971-2000 year period).

Collection of Tissues and Organs

Sexually mature frogs of either sex were collected monthly throughout the study period. Within 48 hr of capture, each animal was weighed to the nearest 0.01 g before sacrified and gonads were also weighed after sacrified. The frogs were anesthesized in ice bath for 5-10 minutes or until they became immobile. Then the spinal cord were pitched and abdominal cavity were opened. The gonads were carefully removed from the body cavities, then they were blotted and weighed to the nearest milligram. The removed gonads were utilized for following purposes; gonadal weight, gonadosomatic index (calculated in propotion of gonadal weight to body weight), external morphology, histological and ultrastructural investigations.

Procedures for Morphological Studies of Ovaries

External Morphological Study

In order to examine the external appearance and to classify various stages of oocyte, portions of fresh ovaries were segregated by dipping in 0.2% collagenase for a few minutes with the aid of physical tearing until individual oocyte separating from each other as much as possible. Then the oocytes were classified into various stages mainly based on their size and color under stereomicroscope.

Histological Studies

For light microscopy, routine haematoxyline and eosin staining techniques(H&E) were applied for the detection of substances in the oocytes. The specimens were fixed in Bouin's solution. Conventional paraffin technique was applied to these specimens. Serial section of specimens were sliced at 5-6 um in thickness, thenthey were stained with haematoxyline and eosin. Finally, they were mounted with Balsum and examine the stage of oogenesis under light microscope.

Ultrastructural Studies

For transmission electron microscopy, the specimens were prefixed in 3 % glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 at 4°C, and washed in the same buffer-fixative sollution. Thereafter, they were postfixed in 1% osmium tetraoxide(OsO4) in 0.1 M cacodylate buffer at 4°C for 1 hour. Araldite was applied for embedding. In case of the large yolk ooytes, Spurr with low viscosity was used instead of Araldite. The thin sections were cut by ultramicrotome and stained by uranyl acetate. The stained ultrathin sections were observed under the transmission electron microscope.

Preparations of Testes for Light and Electron Microscopic Studies

Histological Studies

The testes were processed in the same manner as described in section histological studies for ovaries.

Ultrastructural Studies

The testes were processed in the same manner as described in section ultrastructural studies for ovaries.

Seasonal Spermatogenetic Activities

The removed testes were weighed in order to analyse the difference of the testes weight in relation to seasonal differences. These results were utilized for GSI. Parafin embedded testes were sectioned at 5-6 um in thickness and stained with Harris's haematoxylin and eosin. The mean diameter of 15 seminiferous tubules and assessment of spermatogenetic activity were measured and analysed. Sexual maturity of males was based on the presence of spermatogenetic activity. The size of the Leydig cell nucleus was measured under 400x magnification. The number of Leydig cells lining between the seminiferous tubules was counted directly from photographs taken from tubule cross sections (Yoneyama and Iwasawa, 1985). Spermatogenetic cycle was determined by two phases:

spermatogenesis and spermiogenesis. The spermatogenetic activity in different seasons, then, will be compared in relation to the amount of rainfall.

Assessment of Spermatogenetic Activity

Germ cells in seminiferous tubules of frogs were organized into germinal cysts or cell nests at specific stage of differentiation. The stages of spermatogenesis were identified according to the method used by Rastogi *et al.*, 1976. Randomly selected cross sections (15) of seminiferous tubules (five cross sections/animal, three animals/month) were observed under microscope to evaluate the spermatogenetic progress. Five classes were identified as follows; primary spermatogonium, secondary spermatogonium, primary spermatocyte, secondary spermatocyte, and spermatid. Spermiogenesis was recorded by the extent of spermatozoa inside the seminiferous tubules. The proliferation of Leydig cells in the interstitial tissues was recorded.

Females

Sexual maturity of females was based on the presence of enlarged ovarian follicles or enlarged oviducts.

Assessment of Ovarian Follicles in the Ovary

The ovaries were fixed in Bouin's solution. Ovarian diagnoses were made under dissecting microscope equipped with measuring ocular. First, the surface of the ovary was observed to estimate the number of small unpigmented previtellogrnetic oocytes as well as the darkly pigmented atretic oocyte. Then, the sac-formed ovary was opened by pointed forceps to expose the interior of the ovary for evaluation the number of vitellogenic oocytes. The functional status of the ovaries was assessed in adult female frogs collected throughout the year. Normally several functional states of the ovaries could be found simultaneously within frogs, ranging from ovaries with previtellogenic state to all stages of vitellogenic growth of a complement of oocytes to ovaries with full-grown oocytes and to the postspawning state. The seasonal ovarian cycle, then, was compared in relation to the amount of rainfall.

Places and Duration

Places

The histological studies were carried out in laboratory of vertebrate zoology, Department of Zoology, Kasetsart University, and in Electron Microscopic Unit, Department of Anatomy, Faculty of Science, Mahidol University, Bangkok.

The median-striped burrowing frogs were collected in the dry dipterocarp forest, Nongteng-Chakkarat National Reserved Forest, Chalerm Prakriat District, Nakorn Rachsima Province.

Duration

The duration of this study covered the period from November 1999 to October 2000.

RESULTS

Classification of Spermatogenic Cells

Histological sections of testis showed that they contain numerous seminiferous tubules which are bounded by outer basal lamina and elements of interstitial connective tissues. Each seminiferous tubule consists of 2 groups of cells, sustentacular cells or folliculo-Sertoli cells and spermatogonial cells. The interstitial areas are filled with specialized endocrine cells called Leydig's cells, including blood and lymphatic vessels. The male germ cells in seminiferous tubules of median-striped burrowing frogs, *K. mediolineata*, are organized into germinal cysts or cell nest. They can be distinguished into 12 stages based on nuclear characteristics and sizes (Fig.8).

1. Primary spermatogonia

Primary spermatogonia are the first stage germ cells in seminiferous tubules. They generally locate adjacent to basement membrane of seminiferous tubule wall. The cells are large and round in shape and also having round or oval nuclei with fine and mostly euchromatic material. The size of the nucleus is about 10-12 um. Each nucleus possesses one or two nucleoli which are very prominent. The cytoplasm is generally lightly stained. (Fig. 8B).

2. Secondary spermatogonia

Secondary spermatogonia are also round but the cells are smaller when comparison to primary spermatogonia in which nuclear diameter is about 6-8 um. The nuclei contain small blocks of heterochromatin distributed along the nuclear envelopes. The nucleoli still remain prominent. Each group of secondary spermatogonia usually consists of 2 or 4 cells surrounded by the processes of follicle cells which still lie close to the basement membrane (Fig. 8B).

3. Leptotene spermatocytes

These cells are larger than secondary spermatogonia and have round shape (Fig. 8A). They have large round nuclei with diameter about 7-9 um and having thin rim cytoplasm. The chromatin begin to arrange in loosely blocks which are distributed evenly throughout the nucleus. Nucleoli can not be detected in this stage. Leptotene spermatocytes are located toward the lumen of the seminiferous tubules and seldom touch the basement membrane. Groups of leptotene spermatocytes forms large clusters of more than four cells each which are surrounded by follicle cells.

4. Zygotene spermatocytes

The discriminating features of zygotene spermatocytes are the increased condensation of chromatin blocks and the change in size of the nuclei which become smaller than Leptotene spermatocytes (Fig. 8A).

5. Pachytene spermatocytes

The nuclei of pachytene spermatocytes are still round and about 6-8 um in size. The chromatin become condensed into long and thick cords which are interwined into loops resembling "bouguet pattern". The number of cells in each cluster increases in which each cluster is still enclosed by follicular cells (Fig. 8B).

6. Diplotene spermatocytes

The general features of the cells in this stage are similar to pachytene spermatocytes, however, the heterochromatin cords become denser and larger, thus the nucleus appears smaller than in pachytene spermatocytes. The size of the nuclei is about 5-6 um. Diplotene spermatocytes are also fewer in number in comparison to pachytene spermatocytes (Fig.8B).

7. Diakinetic and metaphase spermatocytes

The cells in these stages show thick chromosomes and lie close together in Diakinetic. They later move to the equatorial region in metaphase spermatocytes when the

nuclear boundaries disappear. They are so few and transient that they are rarely distinguished within the seminiferous tubules (Fig. 8B).

8. Secondary spermatocytes

They have dense blocks of heterochromatin which distribute in cartwheel or clock-faced pattern within the nucleus which are arising from the coarse clumping of chromatin along the nuclear envelope whereas nuclear diameter becomes decreasing (Fig. 8A).

9. Early spermatids

They are markedly decreased in size but still have round nuclei which are also reduced in size to approximately 4-5 um in diameter and located eccentrically within the cells. The nuclei are deeply stained over one-half while the rest appears very lightly stained (Fig. 8B).

10. Middle or round spermatids

The chromatin in this stage become evenly condensed and deeply stained throughout the nuclei. Each nucleus tends to be oval in shape (Fig. 8B).

11.Late spermatids

The nucleus of Late spermatids is reducing in size and begins to elongate. Chromatin become completely condensed throughout the nucleus. They locate close to the lumen of the seminiferous tubules and still group together in clusters (Fig. 8B).

12. Spermatozoa

Mature spermatozoa have highly elongated head and tail. The head contains an elongated nucleus with completely condensed chromatin which are deeply stained. Heads of several spermatozoa tend to arrange in array for embedding in cytoplasm of Sertoli cells while their tails point toward the lumen of seminiferous tubules (Fig. 8A, B).

Ultrastructures of Various Stages of Spermatogenesis

Primary Spermatogonia

The primary spermatogonia are large round cell, 14-16 um in diameter. They have large nucleus which contains entirely euchormatin material and very prominent nucleoi. Mitochondria tend to congregate in one pole of the cell together with centriole. Later, primary spermatogonia decrease in size and their nuclei begin to show thin rim of heterochromatin along the nuclear envelopes (Fig. 9).

Secondary Spermatogonia

The secondary spermatogonia are smaller than primary spermatogonia and still maintain round shape, their diameter about 10-11um. There is increasing amount of heterochromatin in the forms of small blocks attached to the nuclear envelopes, and some scattering throughout the nucleus (Fig. 10). The nucleoli are still prominent.

Leptotene Spermatocytes

Leptotene spermatocytes are larger than secondary spermatogonia, their diameter about 10-12 um. The chromatin begin to condense into large heterochromatic blocks that are scattered evenly throughout the round nucleus (Fig. 11A).

Zygotene Spermatocyte

Zygotene spermatocytes have approximately the same size as Leptotene spermatocytes but their nucleus show increasing condensation of heterochromatin blocks, and characteristically, there are synapsed regions of the chromosomes called "synaptonemal complexes" which distribute among the blocks of heterochromatin. The synaptonemal complex appears as tripartite structure with two paralleled dark thick lines bisected by thin dense line (Fig. 11B).

Pachytene Spermatocytes

Pachytene spermatocytes can be distinguished from the former stage by distinctly condensed chromatin blocks that may be somewhat elongated into fiber-like (Fig. 12A). This pattern gives rise to the "bouquet arrangement"of chromatids as observed by the light microscope.

Diplotene Spermatocytes

Diplotene spermatocyte resemble pachytene spermatocytes except it is reduced in size and heterochromatic blocks become closely packed and somtimes attached to the nuclear membrane (Fig. 12B).

Secondary Spermatocytes

Secondary spermatocytes can be identified by using the clumping of chromatic blocks into mesh of "X" or "Y" figures (Fig. 13A). The nucleus remains round and undergoes reduction in size to about 5-6 um. Nucleoli are absent at this stage.

Spermatids

Base on the pattern of chromatin condensation, spermatids may be classified into three morphological stages. The early spermatid stage is a round cell with partially dense nucleus. The chromatin condensation occurs over one-half of the nucleus and the remainting area appears lightly stained (Fig. 13B). The nucleus is still round and locate centrally, and acrosome has not yet developed. The middle (or round) spermatid stage shows dense granular chromatin that is evenly spread throughout the ovoid nucleus. Acrosome may start appearing as flat structure covering one pole of the nucleus (Fig. 13B). The cytoplasm is relatively clear in comparison with cells of earlier stage and cytoplasmic organelles become sparsely. Late spermatid has an extremely dense elongated nucleus. Chromatin become completely condensed throughout the nucleus due to the tight packing of chromatin granules (Fig. 14B, 16B). The cytoplasm becomes highly vacuolated and starts degenerating.

Spermatozoa

Spermatozoa are recognized by their characteristic elongated shape (Fig. 14A, 15). The nuclei appear as elongated cylinders and slightly tapered at the anterior end. The nuclei appear extremly dense and contain very few intranuclear vacuoles. The anterior portions of the nuclei are covered by the acrosomes. The spermatozoa have abundant cytoplasm in the neck region that connect the nucleus to the middle piece. Mitochondria in the middle piece are quite abundant and they are not arranged into helical pattern like in mammals.

Folliculo-Sertoli Cells

Each primary spermatogonium is completely enclosed by the follicle cells. These follicle cells which later mature into secretory Sertoli cells do not possess ultrastructural features characteristic of steroidogenic cells in the intitial stages. Follicle cells are distinguished by their ellipsoidal nuclei containing thin rim of heterochromatin along the nuclear envelopes with the rest of chromatin appears fairy dense (Fig. 9B). The cytoplasm contains sparse organelles and branches into long processes that embrace clusters of germ cells. On the other hand, Sertoli cells are characterized by large and irregular shaped nuclei (Fig. 16,18). Their chromatin are mostly in euchromatic form and the nucleoli are very prominent. The cytoplasm contains an assortment of organelles (e.g. rough and smooth endoplasmic reticulum, mitochondria). Clusters of spermatozoa have their heads lodged within the apical cytoplasm of Sertoli cells.

Leydig Cells

Leydig cells occupy intertubular spaces. They have spindle or elongated shapes with large round nuclei containing mostly euchromatin. The dominant feature of these cells is the presence of fairy large lipid droplets and numerous small dense granules throughout the cytoplasm. Some Leydig cells contain large lipid inclusion which may be transformed into lipofusion-like particles (Fig. 17).

Characteristics of Staging Oocytes

The multilobed ovaries of adult frog contain various stages of oocytes which can be classified into six stages based on size, color, and histology.

Stage 1 Oocyte: Previtellogenic Stage

Under the stereomicroscope, the stage 1 oocyte can be identified by translucent cytoplasm with diameter ranging from 50-300 um (Fig. 19). The nucleus is clearly visible through the cytoplasm and occupies large portion of the oocyte. Also visible with stereomicroscope is a densely packed mitochondria (Fig. 20C). At light microscopic level, the cytoplasm of the previtellogenic stage 1 oocyte appears heavily basophilic. In addition, it also acquires smooth nuclear membrane and nucleoli of various sizes (Fig. 22A, 23).

Stage 2 Oocyte: Previtellogenic Stage

This stage acquire a characteristic white color. Its size ranges from 300-450 um (Fig. 19). The nucleus becomes inconspicuous under stereomicroscope. Hence, the translucent stage 1 oocyte can be easily separated from the dark opaque stage 2 oocyte. Histologically, the presence of few rows of peripheral vacuoles (cortical alveoli) seems to be the most predominant characteristics of stage 2 oocyte (Fig. 22A, 23A, 24A). In addition, numerous nucleoli which vary in size can be observed in each cell.

Stage 3 Oocyte: Vitellogenic Stage

The stage 3 oocyte appears intensely white. The diameter of stage 3 oocyte is 450-600 um (Fig. 19). Histologically, the number of vacuoles gradually increases, and they become dispersed towards the central area (Fig. 22A, 23A, 24B). Yolk platelets are formed and replace the central vaculoes rapidly. The vitelline envelope also becomes conspicuous under the follicle cells. Pigmented granules first appear in this stage and are located at the periphery of the oocyte. The nucleus of this stage possesses convoluted nuclear membrane and numerous nucleoli.

Stage 4 Oocyte: Vitellogenic Stage

The distinguishing morphological feature is the appearance of pigmentation which is light brown to brown in color. The diameter is about 600-1000 um (Fig. 19). Yolk platelets completely replace the central vaculoes while the remaining vacuoles are located around the periphery of oocyte (Fig. 22A). The nucleus is surrounded by highly convoluted nuclear membrane and contains large number of nucleoli.

Stage 5 and 6 Oocytes: Vitellogenic and Fully Grown Stage or Postvitellgenic Oocytes

Both stage 5 and 6 oocytes have distinguishing feature of polarity, the animal pole and the vegetal pole due to difference in the pigmentation (Fig. 19, 20C). The diameters are about 1000-1200 um and 1200-1300 um respectively. The postvitellgenic stage 6 oocyte has dark brown animal pole and white to cream-colored vegetal pole whereas it appears light brown vegetal pole in stage 5 oocyte. Histologically, the vegetal pole contains large-yolk platelets. The nucleus is eccentrically situated near the animal pole (Fig. 8, 22, 23A, 25). The vaculoes are decreased in number while the yolk accumulation increases. The animal pole in the stage 6 oocyte has only one row of vacuoles on the periphery whereas two or three rows of vacuoles are present in the vegetal pole (Fig. 25C, D).

Ultrastructures of Various Stages of Oocytes

All stages of oocytes are covered with three cellular layers (Fig. 26A). The outer most layer is simple squamous epithelium referred to as surface epithelium. Beneath this is layer containing fibrobasts which secrete ground substance and make collagen fibers. The innermost layer is made up of follicle cells which project their cytoplasmic processes into the periviteline space. In addition, the oocyte coat consists of non-cellular layer, the vitelline envelope, whose fibers are interposed between microvilli extending from the oocyte surface (Fig. 29).

The major portion of the cytoplasm of the stage 1 oocyte abound with ribosome. Within perivitelline space, there are cytoplasmic projection both from follicle cells called follicular processes and from oocyte surface called microvilli . Other cytoplasmic organelles

situated at the peripheral part of cytoplasm include large number of mitochondria, some Golgi apparatus, groups lysosomes (multivescular bodies) and a few lipid droplets (Fig. 26B,C). The nuclear envelope has a smooth contour. Various sized electron-dense nucleoli appear in the nucleus.

In stage 2 oocyte, the most prevalent cytoplasm components are newly developed vacuoles or cortical alveoli on the periphery of the oocyte (Fig. 26B). Some vacuoles are closely associated with lysosomes. The Golgi apparatus is also well developed. In this stage, the vitelline envelope begins to from as isolated bundles of fine filaments within the perivitelline space (Fig. 26B, C). Both the microvilli of the oocyte and the cytoplasmic processes of the follicle cell become longer. The number of elongated mitochondria progressing to spherical shaped mitochondria is increased and these are distributed around the nucleus.

In stage 3 oocyte, there are two major events, the deposition of yolk plaletes and the formation of pigmented granules (Fig. 27). Vacuoles become larger in size. Groups of mitochondria are observed among the yolk platletes of vitellogenic oocytes and pinocytotic activity on the surface of the oocyte is initiated (Fig. 27B, C). Newly synthesized yolk platelets exhibit bipartite characteristics, the central paler compact body which is arranged into crystalline lattice and the peripheral electron-dense portion. Although pigmentation begins in this stage, most oocytes still contain only a few membrane-bound melanin granules called premelanosomes. Nuclear membrane is highly convoluted.

The stage 4 oocyte is characterized by abundant pinocytotic vesicle under the oolemma. More electron-dense main yolk bodies are observed in addition to the newly formed yolk platelets. Groups of mitochondria and lipid droplets are intermingles among yolk. Increasing number of yolk platelets in the perinuclear cytoplasm result in depletion of mitochondria at this region. Highly folded nuclear membrane causes the sacculated nuclear outline.

In the stage 5 oocyte, most pigmented granules migrate toward the animal pole leaving only a few of them at the vegetal pole and perinuclear zone. During this stage, the vacuoles with their transucent content are conspicuous underneath the oolemma (Fig. 29A). Some pinocytotic activity could still be observed. Although the general ultrastructures of the

stage 6 oocyte are quite similar to the stage 5 oocyte, the pigmented granules are completely absent from the vegetal pole. Many of the microvilli are lost from the oocyte's surface (Fig. 28, 29).

Gonadosomatic Index (GSI), Gonadal Morphology, and Reproductive Gonadal Cycle

Females

In winter (November-February) oocytes consisted of small previtellogenic oocytes containing peripherally-located cortical alveoli, the vitelline envelope begins to form as isolated bundles of fine filaments within the perivitelline space. The microvilli of the oocyte become longer. The proliferation of microvilli together with the folding of the nuclear membrane marks the end of the previtellogenic phase of oocyte growth, and is followed by the growth phase. As oocytes growth proceed , exogenous yolk deposition increased so that pinocytotic vesicles and yolk plateletes were evident by the hot season, March-April. Ultrastructurally the follicle cells of the small previtellogenic oocytes have the appearance of fibroblasts and contain very few organelles. In the hot season, the follicle layer becomes hypertrophied, the glandular cells appearance, they develop a prominent endoplasmic reticulum. Mitochondria become more numerous. During the oocyte is in the cortical alveoli formation stage and in the vitellogenesis stagethe heterochromatin is mainly positioned peripherally in the nuclei of the follicle cells, and well developed endoplasmic reticulum and Golgi complexes are present in the cytoplasm.

Seasonal Differences of The Ovarian Cycle

To study this difference, the whole year was divided into three periods characterized by different ovarian functional patterns. The first period include the cold months from November to February which coinciding with the north east monsoons, the period of dry and relatively cold part of the year (Fig. 2). The second period include the months of March and April, the hot season. The third period include May to October, or the period of monsoon rains. Since only females with convoluted oviducts had enlarged ova, I have used the form of the oviducts as a criterion of sexual maturity.

Table 1 Seasonal differences in the average body weight, testicular weight, GSI, seminiferous tubules and Leydig cell nucleus diameter in adult *K. mediolineata*.

month	N[1]	Body weight[2] (g)	Testicular weight[2] (g)	GSI	Diameter (u) of	
					Seminiferous tubules[2]	Leydig cell nucleus[2]
1999						
November	10	24.49±1.4	0.019±0.04	0.07	125.8±50.8	3.08±0.24
December	10	22.14±2.0	0.019±0.06	0.08	126.9±33.0	2.47±0.25
2000						
January	8	20.26±1.3	0.020±0.06	0.09	130.1±16.7	3.76±0.09
February	8	19.38±1.3	0.020±0.11	0.10	133.8±1.1	3.50±0.10
March	7	20.21±1.2	0.022±0.07	0.10	150.1±21.2	3.84 ±0.08
April	10	20.94±0.9	0.027±0.09	0.12	170.9±47.0	4.53±0.23
May	10	22.39±1.5	0.028±0.02	0.12	175.9±38.3	5.38±0.20
June	10	23.06±1.5	0.026±0.13	0.11	166.2±26.0	4.69±0.23
July	10	22.42±1.2	0.027±0.07	0.12	170.6±35.2	4.72±0.22
August	10	23.36±2.5	0.027±0.12	0.11	157.4±42.3	4.97±0.21
September	10	25.28±1.9	0.021±0.04	0.08	150.3±26.0	3.72±0.22
October	10	25.10±1.2	0.019±0.03	0.07	131.2±20.4	3.44±0.13

[1]Number of animals

[2]The data are expressed as the mean ±SE

Concerning seasonal differences, it is observed that there is no stage 6 oocytes occuring in ovaries collected during January and April. Most of the oocytes were previtellogenic oocytes, some are stage 3 and 4 (Fig. 20A, 21). In the present study, stage 6 oocytes were presented in the transitional period of the end of the hot season to the early wet season (early May) continued to the period of the wet season (Fig. 20 B,C). In October to December, some ovaries contained stage 6 oocytes, possibly remnants of previous ovarian cycle. Degenerated (or atretic) oocytes (Fig.20A, 21) appeared throughout the year and increased in number during October to February.

Males

The two principal elements of the testis are the seminiferous tubules and the interstitial tissue consisting of closely packed small ovoid Leydig cells.

Seasonal Differences of Spermatogenetic Cycle

Seasonal differences in histological observations of the spermatogenetic activity in the testis of *K. mediolineata* collected monthly from November 1999 through October 2000 are representatively shown in Fig. 30 to 34. All males collected were sexually mature, judging by the presence of sperm production. The spermatogenetic activity showed all stage of spermatogenesis almost of the year. No adults had testes with complete regression: i.e., without cell division. Maximum levels of spermiogenesis and spermiation ocurred during the hot season through the wet season, mainly in the middle of April to August when clusters of sperm attached to Sertoli cells, or free spermatozoa were observed in the lumen (Fig. 30B, 31, 33B). Spermatozoa were greatly reduced in number in October (Fig. 34). The testis in October contains relatively few cell nests most of which are in the spermatogonia stage. Many of the primary stage do not develop further than primary spermatocytes and the maturing spermatids which anchoring to Sertoli cells (Fig. 30C) ; also the unshed free spermatozoa from the previous breeding month (August). However, the active spermatogenetic activity takes place from the cold season to the hot season with the concomitant slightly increase in GSI and diameter of the seminiferous tubules (Table 1) due to rapid increase in the number of cell nests (Fig. 30A). Most of these cell nests transform into spermatozoa during the period of late April to the wet season and in these testes, the seminiferous tubules are filled with spermatozoa (Fig. 30B). Clusters of spermatozoa were

also found in the seminiferous tubules during the cold season (November to February) (Fig. 30A, 32). In the end of the wet season (October) there is a decline in GSI and diameter of the seminiferous tubules due to the evacuation of the spermatozoa.

Sertoli Cell

Sertoli cells are derived from the flattened follicles that surround the primary spermatogonia (Fig. 9B). During meiotic phases of spermatogenesis, and toward the end of spermiogensis, these cells become conspicous Sertoli cells, the nucleus becomes rounded, acquire smooth endoplasmic reticulum, spherical mitochondria with tubular cristae, lipid droplets (Fig. 15A, 16A, 18). Maximum levels of spermiogenesis and spermiation occurred during the hot season through the wet season. In *K. mediolineata* when spermatids are deeply embedded in the cytoplasm of the Sertoli cell, the zone of endoplasmic reticulum was characterized by the presence of dilated membranes and vacuoles which freely interconnected with each other (Fig. 18A). Bundle of spermatozoa can be found embedded in the apical cytoplasm of Sertoli cells (Fig. 15A, 31A).

Leydig Cell Cycle

In this species, well marked seasonal differences is observed in the Leydig cell size, shape, and certain cytological features. The Leydig cells are round in outline and abundant in number in breeding period (Fig. 31B). They exhibit maximum nuclear diameter during May-August which is the breeding period of this species. The nuclei of these, during this period, also contain coarse chromatin granules. Soon after the breeding time in October-November the Leydig cells become elongaed shape (Fig. 30C) and reduced in size (Table1). These cells become less frequent. During this period, these cells possess fine chromatin granules. The Leydig cells remain in this state upto next March. During the next March-April the Leydig cells increase in size and number and become round in outline. The redistribution of the chromatin material take place and the chromatin material now contain once again coarse granules, the characteristic of secretory cells(Fig. 17).

Observations of Reproductive Cycle and Rainfall

Recruitment of oocytes to vitellogenic growth commences in the hot season (March) throught the wet season. Accordingly, frogs with ovaries in late or postvitellogenic oocytes (stage 5-6 oocytes) can be observed during May to December, but must more frequently from the late May to August. This high incidence of late stages coincides with the rainfall, whereas the amplexing occured only in some periods of rainfall associated with soil moisture and surplus water form the temporary pond for amplexing (Fig. 1). In the current study, the mating call was first observed on February 24, 2000 when the moderated rainfall at 13.7 mm. However, there were not any temporary pond and the amplexing did not occure. On May 21, 2000 there was the heavy rainfall (88.2 mm.), the mating call and amplexing were observed at the temporary breeding pond and the tadpoles appeared in June. These breeding behavior can be observed again in the late August which very heavy rainfall. The tadpoles appeared in September, and disappeared in the middle of October.

Figure 3 Dorsal view of median-striped burrowing frog, *Kaloula mediolineata* .

Figure 4 Ventral view of male median-striped burrowing frog, *Kaloula mediolineata*, showing black chin.

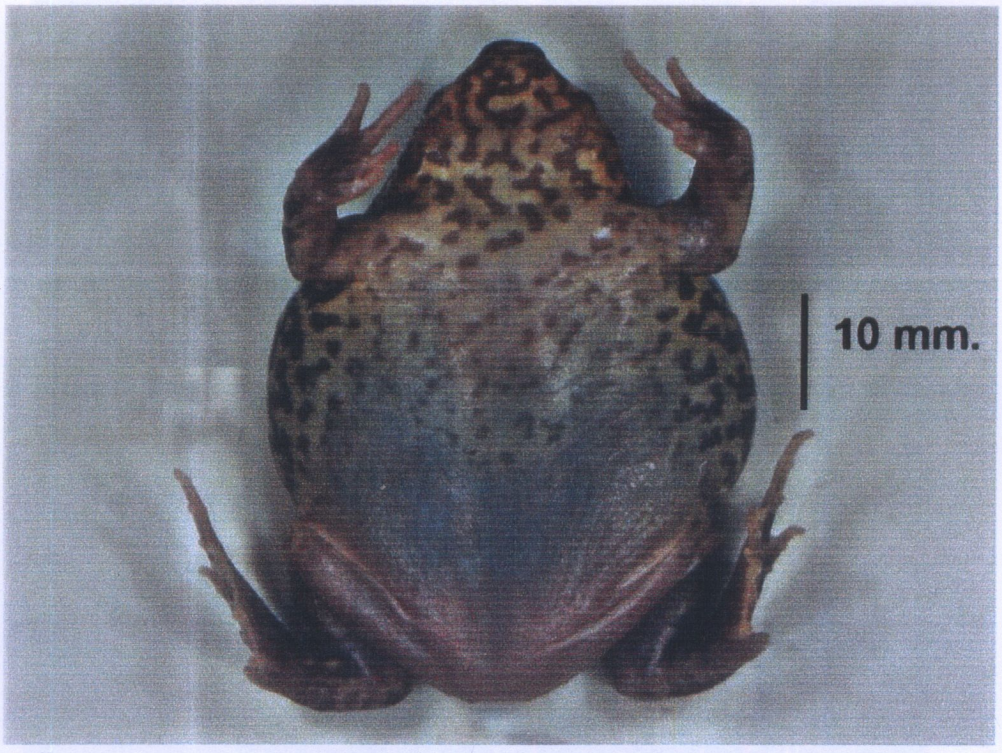


Figure 5 Ventral view of female median-striped burrowing frog, *Kaloula mediolineata*, showing reticulate chin.

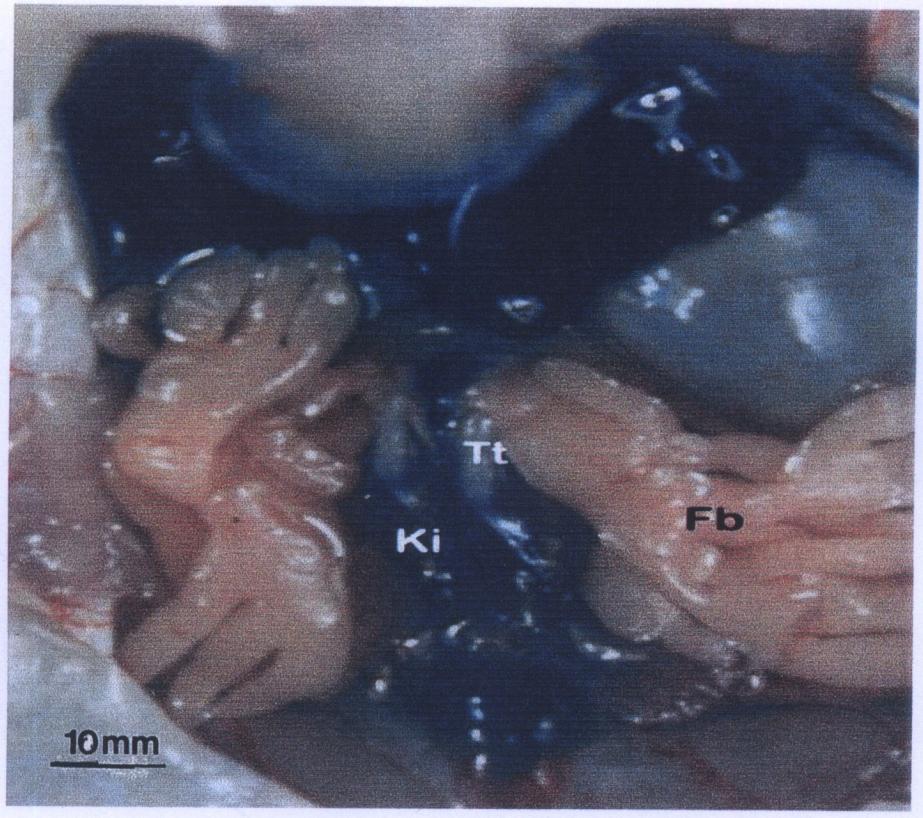


Figure 6 Testis and their associated organ of fully mature frog.
Tt= testis , Fb= fat body , Ki= kidney

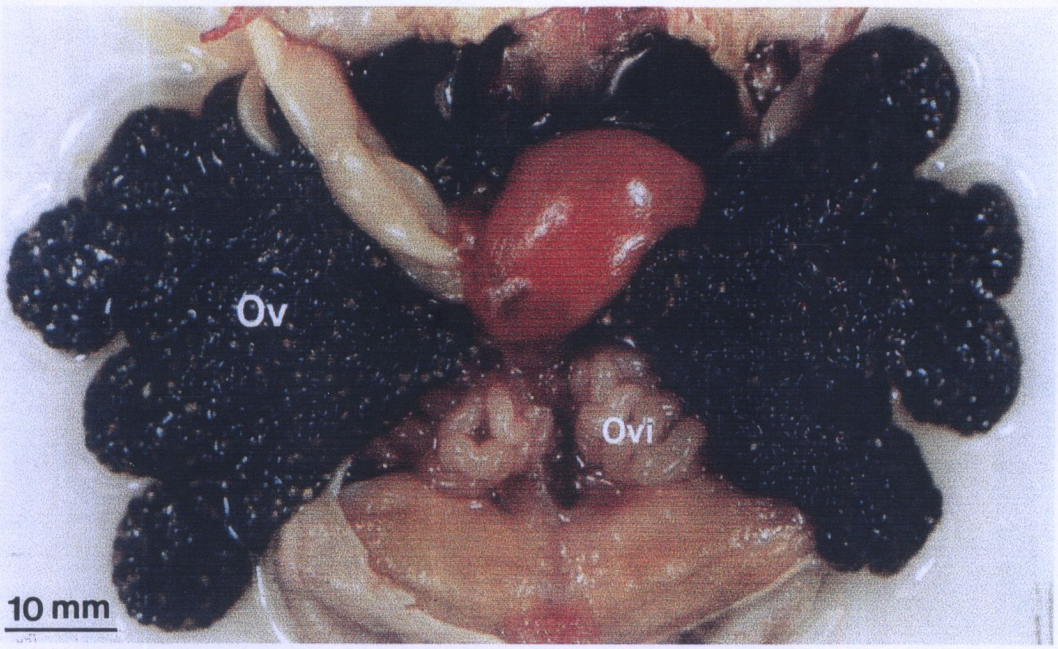


Figure 7 General characteristics of female reproductive system in fully mature frog.
Ov = ovary , Ovi = oviduct

Figure 8 Light micrographs of H&E stained testes. X400.

A,B = Various stages of male germ cells.

ISPG = primary spermatogonia

IISPG = secondary spermatogonia

LSc = leptotene spermatocyte

ZSc = zygotene spermatocyte

PSc = pachytene spermatocyte

DSc = diplotene spermatocyte

MSc = metaphase (diakinesis) spermatocyte

IISPC = secondary spermatocyte

Est = early spermatid

Rst = middle (round) spermatid

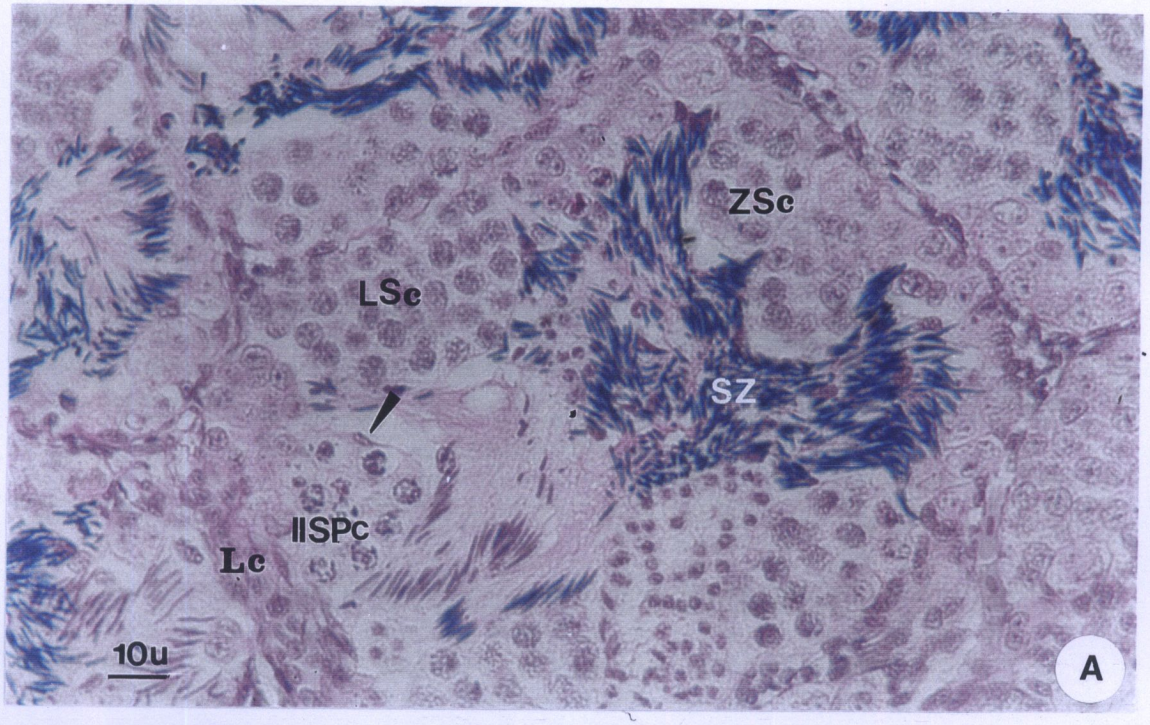
LSc = late spermatid

SZ = spermatozoa

SB = sperm bundle

Lc = Leydig cell

Arrowheads = follicle cells


ZSc
LSc
SZ
IISPc
Lc
10u
A

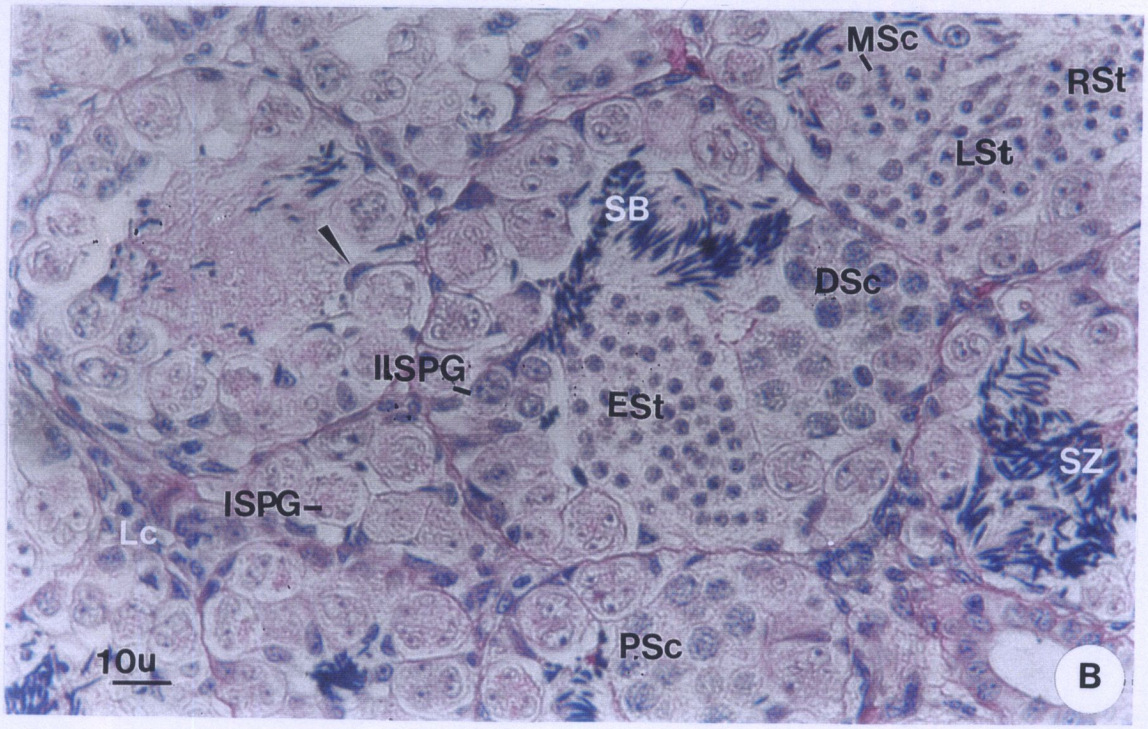

MSc
RSt
LSt
SB
DSc
IISPG
ESt
SZ
ISPG
Lc
10u
PSc
B

Figure 9 Transmission electron micrographs showing primary spermatogonia.

A: Primary spermatogonia showing heterochromatin block (Hc), prominent nucleolus (No), mitochondria (Mi), and lysosome (Ly). X3500.

B: Showing a single large primary spermatogonia enclosed by a layer of thin Sertoli cytoplasmic process (arrowheads). Sertoli (follicle) cell (SC). X3500.

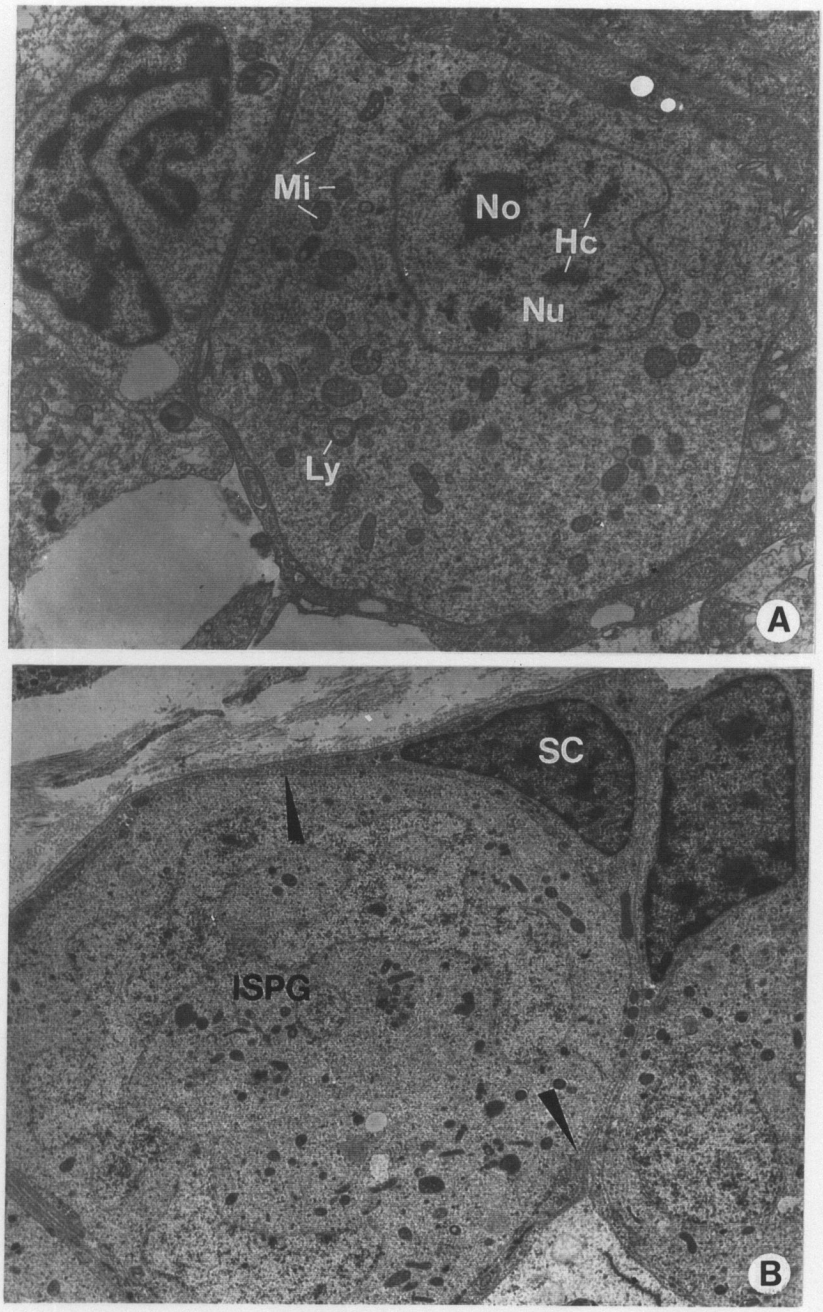

Mi
No
Hc
Nu
Ly
A
SC
ISPG
B

Figure 10 Transmission electron micrographs showing secondary spermatogonia.

A: Secondary spermatogonia showing heterochromatin block (Hc), prominent nucleolus (No), mitochondria (Mi), and lysosome (Ly). X5000.

B: Higher magnification of secondary spermatogonia showing thin strip of heterochromatin (Hc) scattering throughout the nucleus. Nu = nucleus; No = nucleolus. X8000.

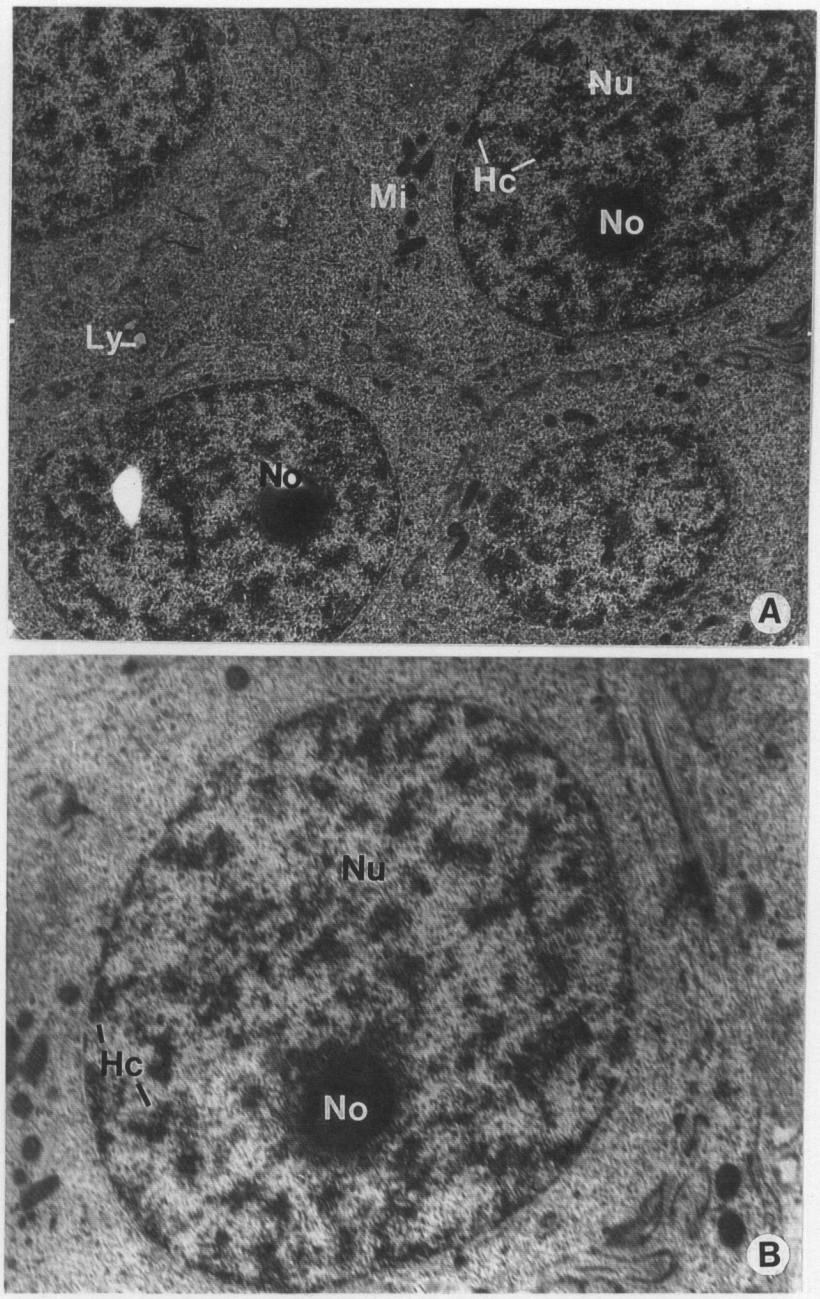

Nu
Hc
Mi
No
Ly
No
A
Nu
Hc
No
B

Figure 11 Transmission electron micrographs of leptotene and zygotene spermatocyte.

A: Leptotene spermatocyte, showing the beginning of chromatin condensation (Hc).

Mi= mitochondria. X3500.

B: Zygotene spermatocyte, showing dense heterochromatin blocks (Hc) with strips of synaptonemal complex (Sy). X5000.

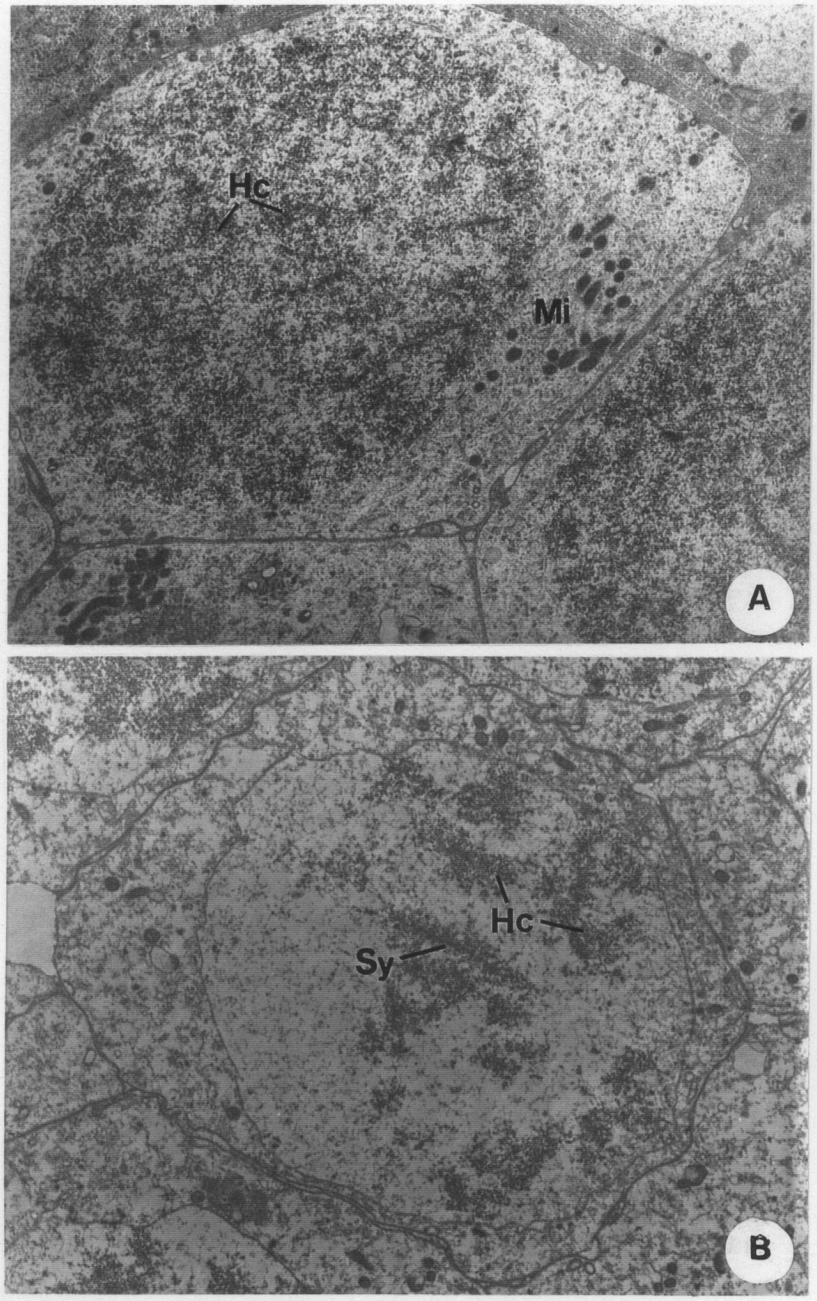

Hc
Mi
A
Hc
Sy
B

Figure 12 Transmission electron micrographs of pachytene and diplotene spermatocytes.
A: Pachytene spermatocyte with increasing condensation of heterochromatin (Hc). X10000.
B: Diplotene spermatocyte, showing increasingly dense heterochromatin block (Hc). X5000.

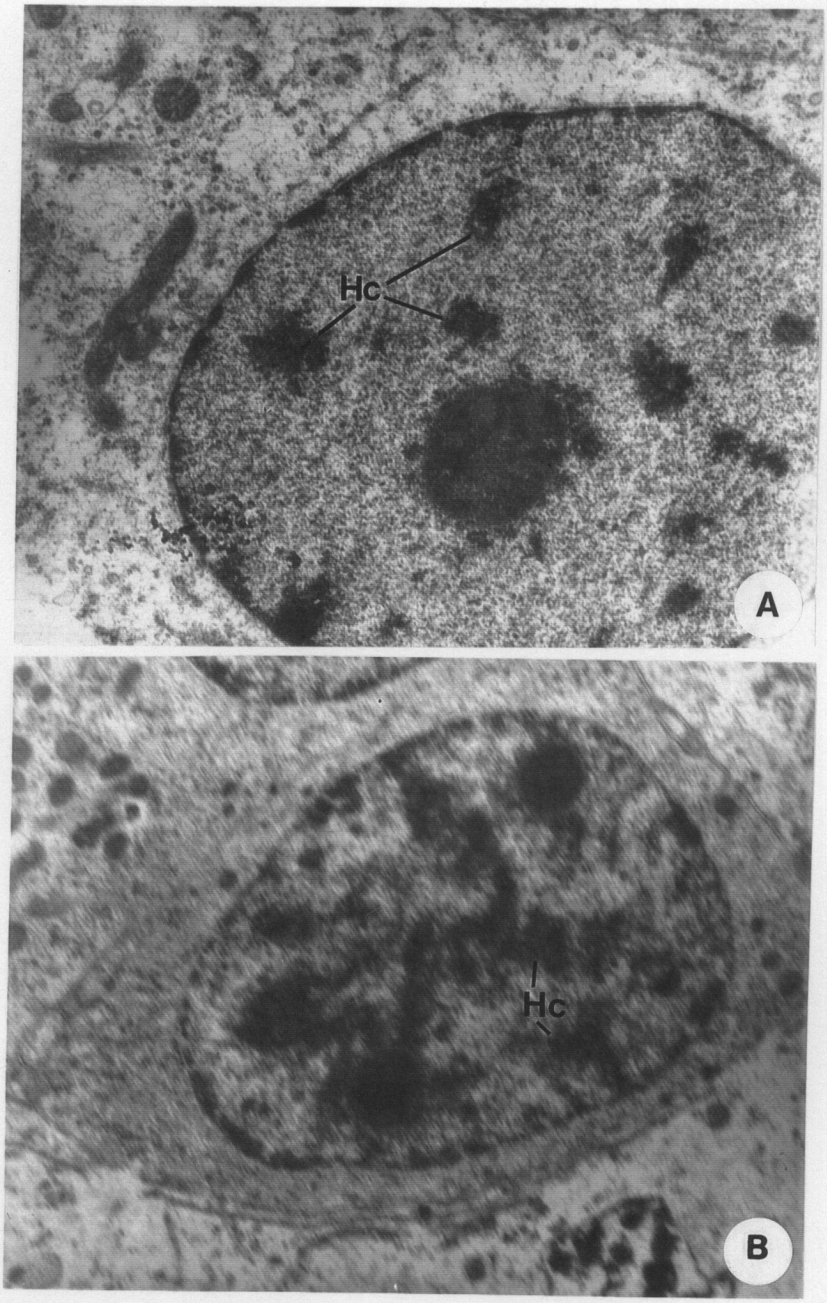

Hc
A
Hc
B

Figure 13 Transmission electron micrographs of secondary spermatocyte and spermatids.
A: Secondary spermatocyte, showing heterochromatin (Hc) in X or Y figures. Mi= mitochondria. X8000.
B: Spermatids showing condensation of heterochromatin (Hc) throughout the nucleus. Mi= mitochondria. X5000.

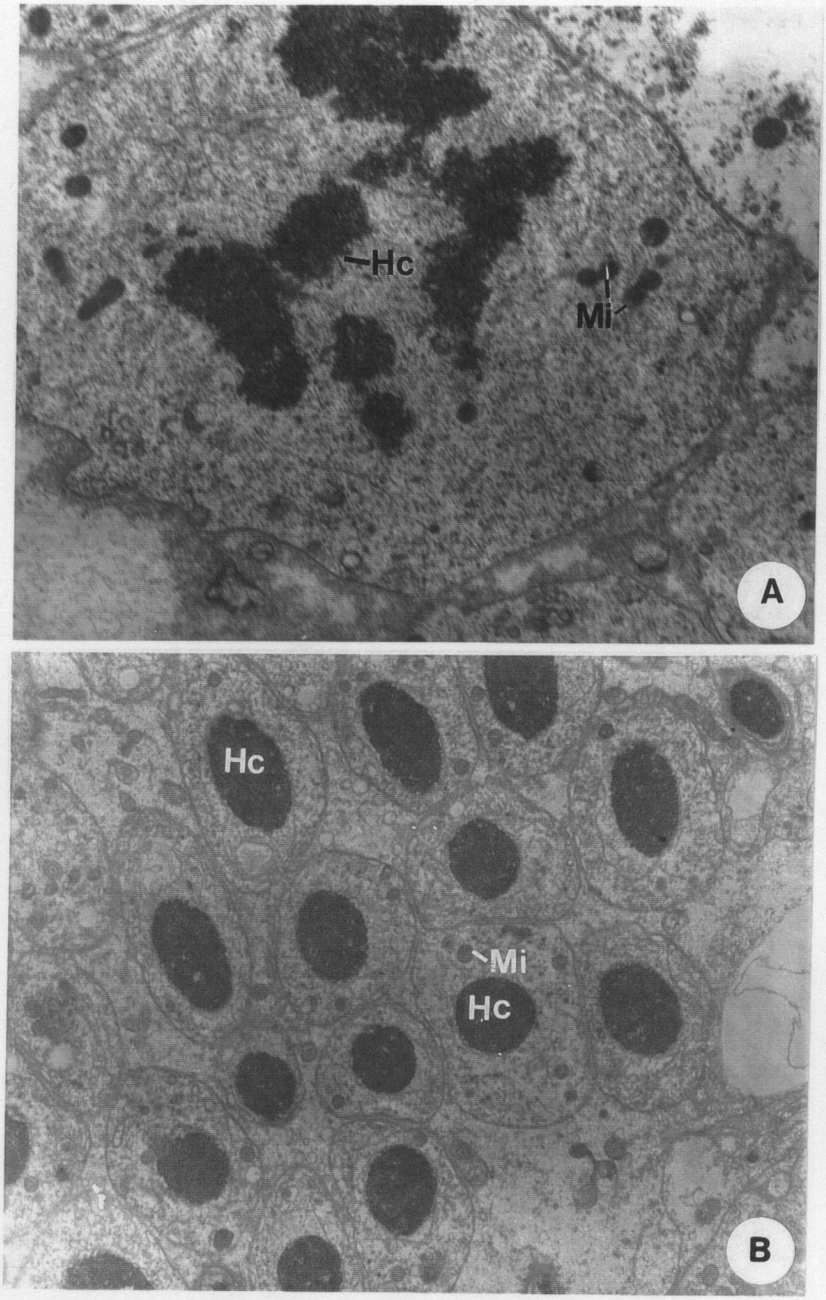

Hc
Mi
A
Hc
Mi
Hc
B

Figure 14 Transmission electron micrographs of early and mature elongating spermatids.
A: Early elongating spermatids individually embedded in Sertoli cytoplasm. Elements of the smooth endoplasmic reticulum (SER) are often closely applied to the surface of lipid droplet (Li) existing in Sertoli cell. Sc=Sertoli cell nucleus. X5000.
B: Mature elongating spermatids embedded in Sertoli cytoplasm. Nu= nucleus; Mi= mitochondria; SC= Sertoli cell nucleus. X3000.

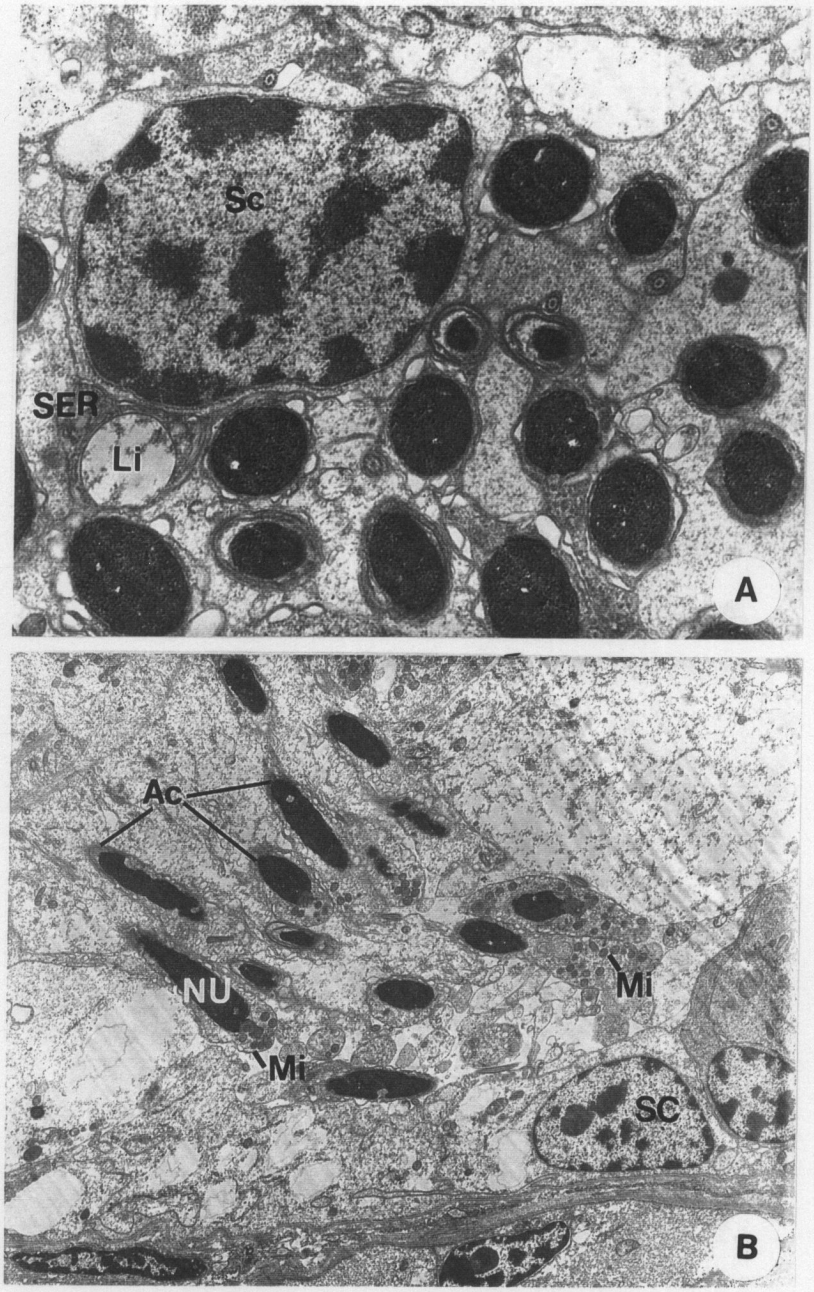

Sc
SER
Li
A
Ac
NU
Mi
Mi
Sc
B

Figure 15 Transmission electron micrographs of early spermatozoa.

A: Early spermatozoa individually embedded in Sertoli cytoplasm. Maximum levels of spermiogenesis and spermiation occurred during the end of the hot season through the wet season. At the end of spermiogenesis, Sertoli cell nucleus (Sc) assume a more rounded shape. SZ= spermatozoa; Nu= nucleus; Ac= acrosome; S= subacrosomal space; Mi= mitochondria. X12000.

B: Each early spermatozoa occupies an individual races within the sheet of Sertoli cytoplasm (arrowhead). X15000.

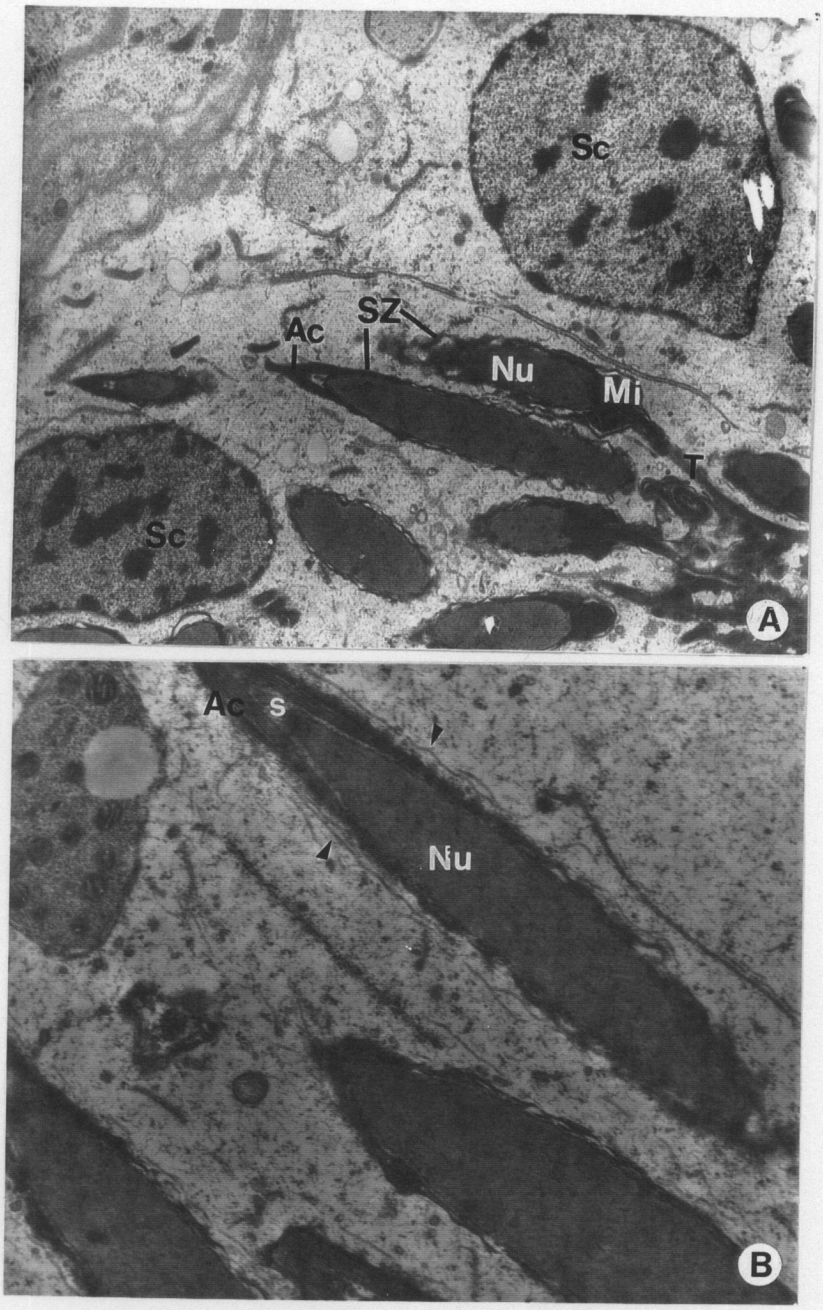

Sc
SZ
Ac
Nu
Mi
T
Sc
A
Ac
S
Nu
B

Figure 16 Transmission electron micrographs of elongating spermatids.

A: Mature elongating spermatid showing the nuclei (Nu) are elongated and dense. Mitochondria (Mi) are tightly packed at the base of nucleus. Ax=axoneme. Maturing spermatid with vacuolized cytoplasm (arrowhead) are indicated. X8000.

B: Developing midpiece and tail, showing mitochondria (Mi), axoneme (Ax), centriole and vacuole (Va). X20000.

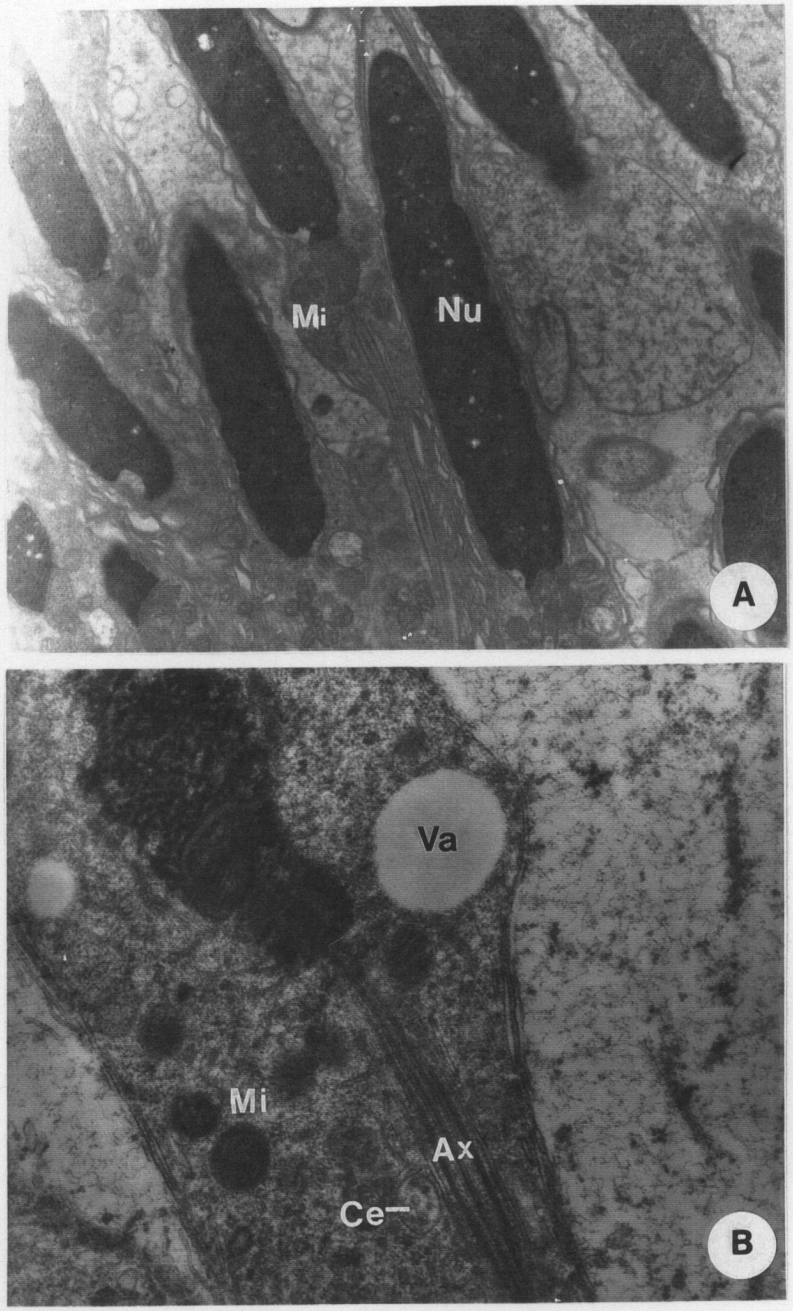

Mi
Nu
A
Va
Mi
Ax
Ce
B

Figure 17 Transmission electron micrographs of Leydig cell.

A. Leydig cell during the wet season, showing large lipid droplets (Li), mitochondria (Mi). Nu= nucleus. X17000.

B. Higher magnification of Leydig cell during the wet season, showing mitochondria (Mi) with tubular cristae and dense granules in their cytoplasm. Nu= nucleus. X40000.

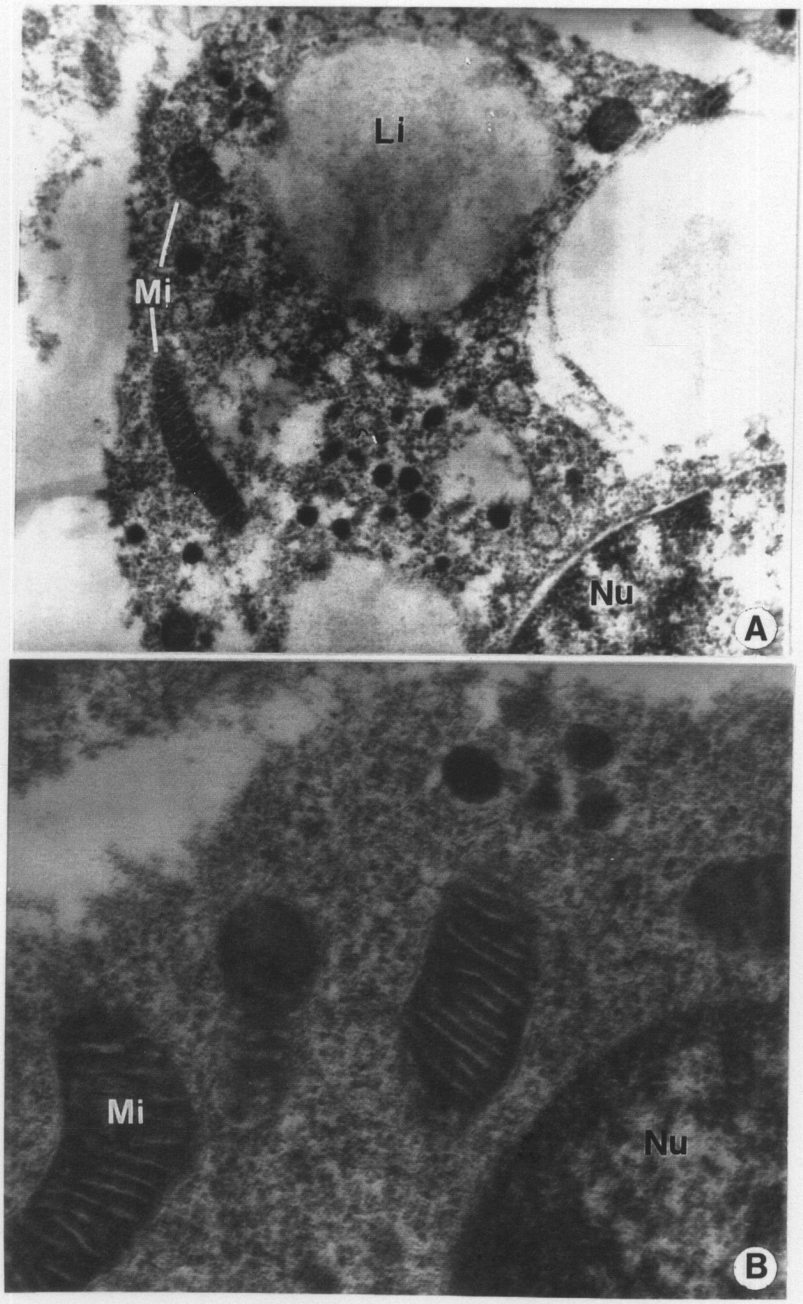
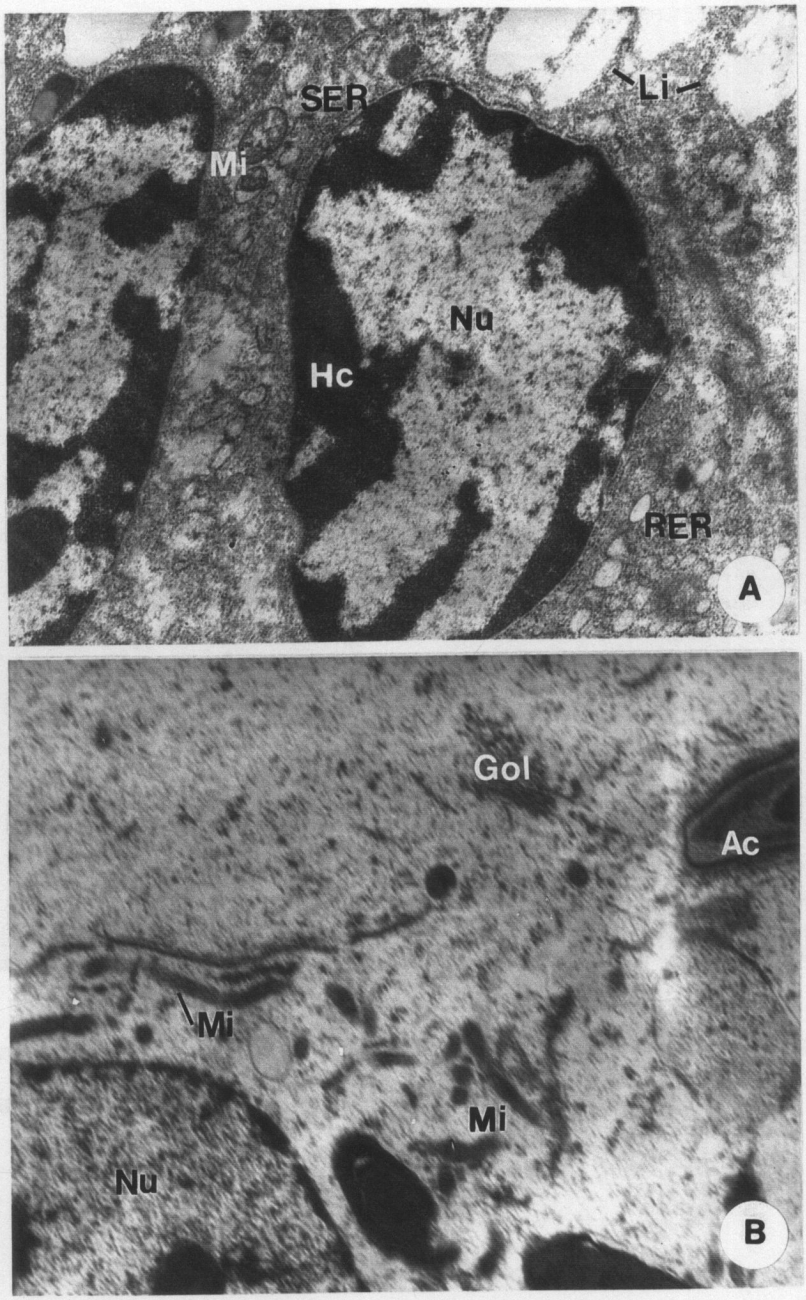

Li
Mi
Nu
A
Mi
Nu
B

Figure 18 Transmission electron micrographs of Sertoli cells.

A: With the progression of spermatogenesis, Sertoli cells hypertrophy, accquire smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER) spherical mitochondria (Mi) with tubular cristae, lipid droplets (Li). The nucleus (Nu) also enlarges and assumed a round shape but contains large clumps of heterochromatin(Hc). X15000.

B: Sertoli cell showing cytoplasm containing small area of golgi complex, mitochondria (Mi) and acrosome (Ac) of spermatozoa. Nu= nucleus. X10000.


SER
Li
Mi
Nu
Hc
RER
A
Gol
Ac
Mi
Mi
Nu
B

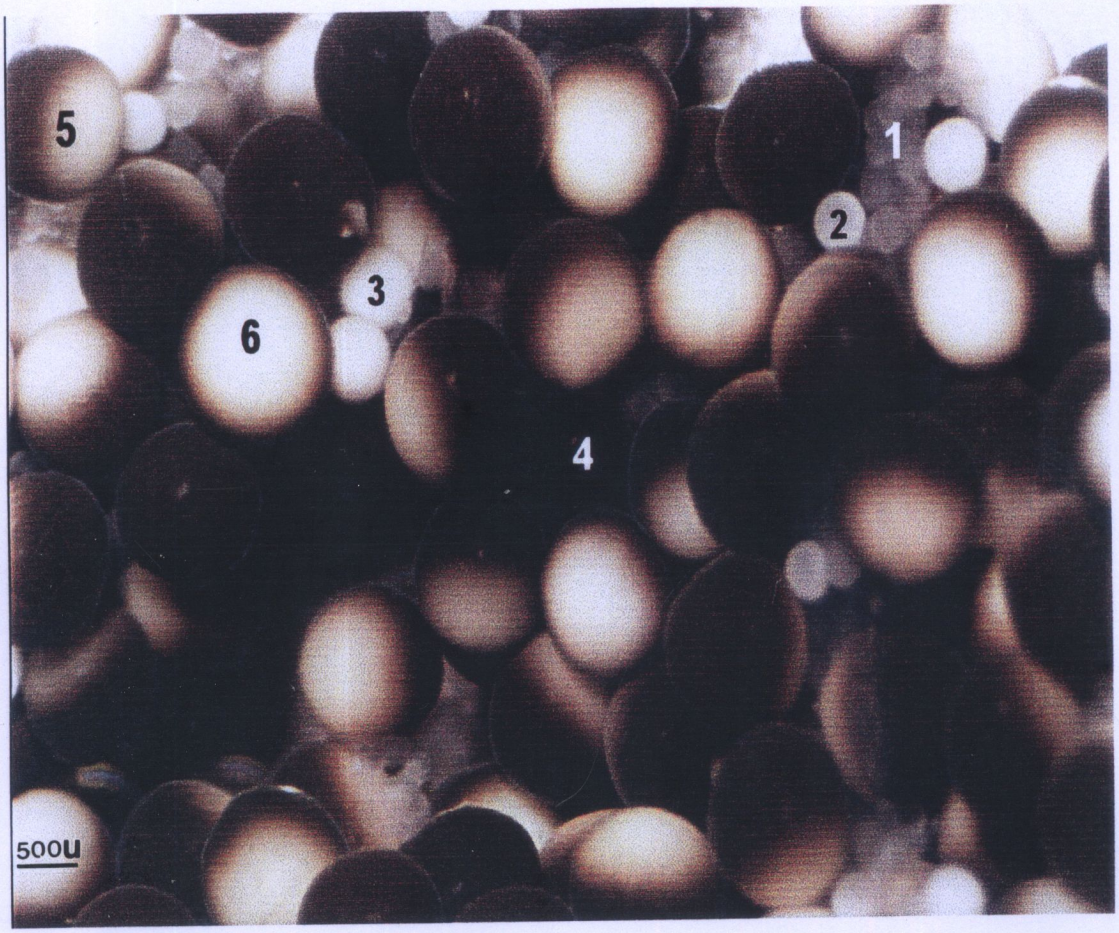


Figure 19 Stereomicrograph of ovarian fragments showing randomly arranged oocytes at stages 1-6.

Figure 20 Stereomicrographs of ovarian fragment in different seasons.

A. Ovarian fragment in hot season (in March) showing small previtellogenic (stage 1, 2) oocytes and early vitellogenic (stage 3) oocytes. Various size of degenerated (D) or atretic oocytes are indicated.

B. Ovarian fragment in hot season (in April) showing small previtellogenic (stage 1, 2) oocytes, early vitellogenic (stage 3) oocytes, and larger vitellogenic (stage 4-6) oocytes.

C. Ovarian fragment in wet season (in May) showing small previtellogenic (stage 1, 2) oocytes and postvitellogenic (stage 6) oocytes. Ap= animal pole, vp= vegetal pole, mm= mitochondrial mass.

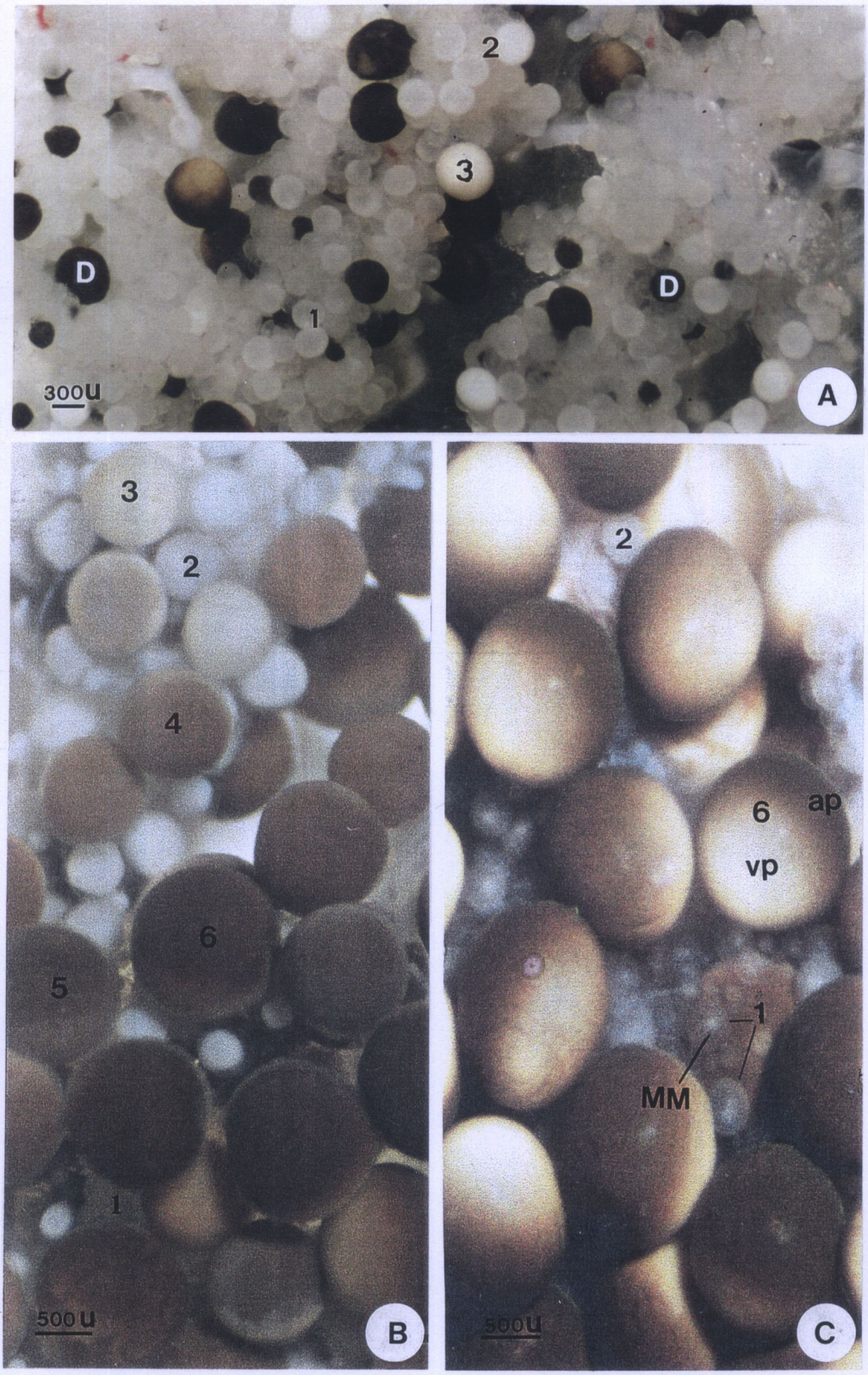

2
3
D
D
1
300u
A
3
2
4
6
5
1
500u
B
2
6
ap
vp
1
MM
500u
C

Figure 21 Stereomicrographs of postspawning ovarian fragment in the cold season. Showing small previtellogenic (stage 1, 2) oocytes and remnant of stage 3,4 oocytes of previous ovarian cycle. Various sizes of degenerated (D) or atretic oocytes are indicated.

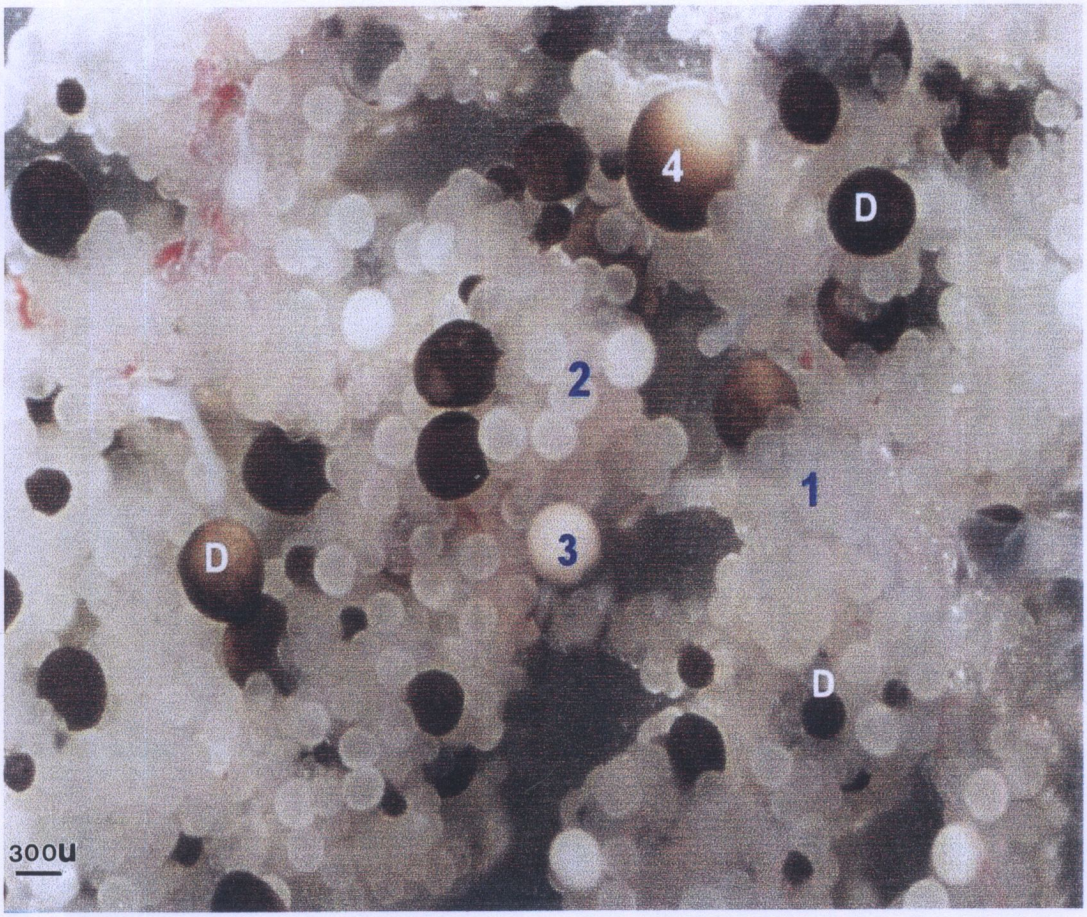

4
D
2
1
D
3
D
300u

Figure 22 Light micrographs of H&E stained oocytes, showing stage 1-6 oocytes. A,B: The vacuoles (Va) are dispersed towards the central vicinity in the early stage 3 oocyte, and later they are replaced by numerous yolk platelets (YP). The centrally-located vacuoles (Va) are completely replaced by yolk platelets during stage 4 oocyte. Distinct polarity including the nuclear movement toward the animal pole occurs in stage 5 and 6 oocytes. AP= animal pole, VP= vegetal pole, VC= vitelline coat. X40.

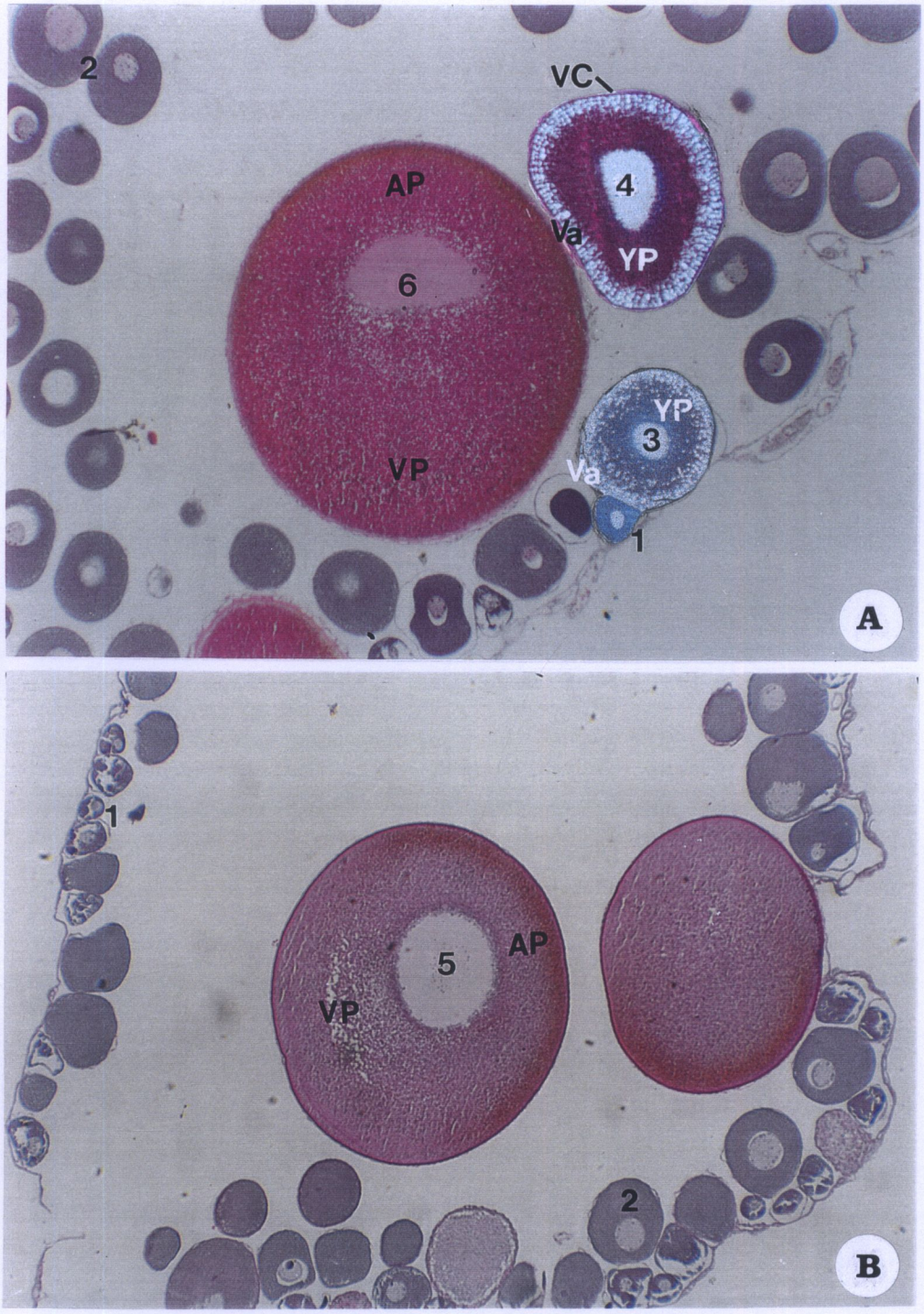
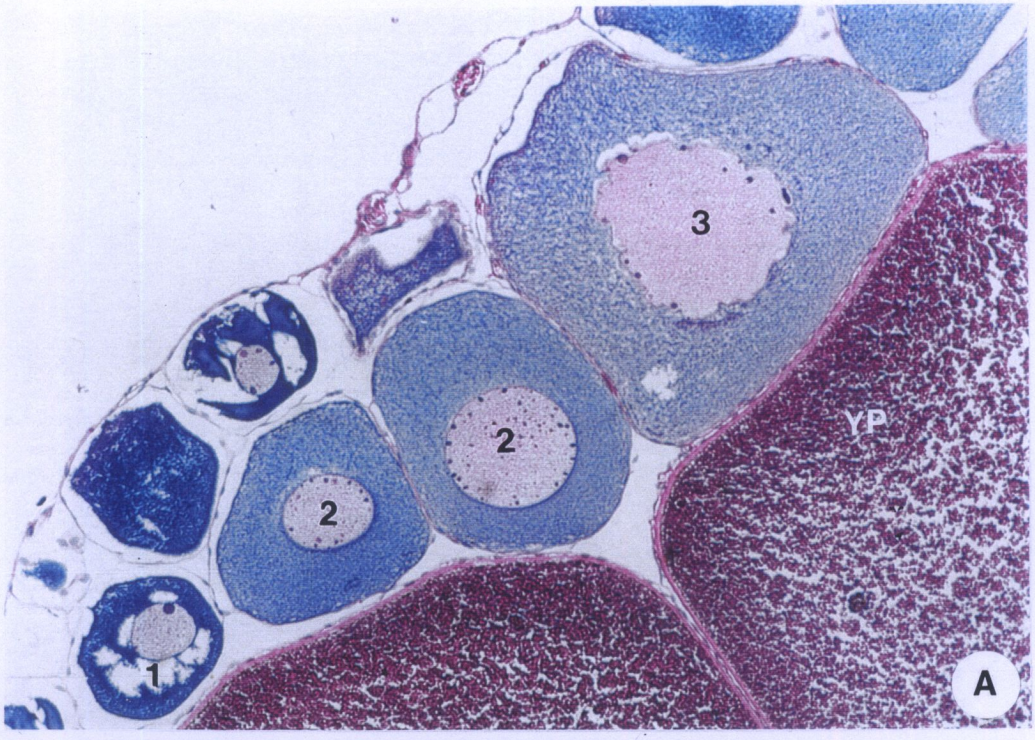

2
VC
AP
4
Va
YP
6
YP
3
VP
Va
1
A
1
AP
5
VP
2
B

Figure 23 Light micrographs of H&E stained oocytes stage 1,2,3 and 6 oocytes. Stage 6 oocyte showing yolk platelets (YP) at the animal pole. Stage 1 oocyte (B) revealing intensively basophilic cytoplasm and smooth nuclear membrane (arrowhead) that become wavy the stage 3 . Nu= nucleus, No= nucleoli, Fc= follicle cells. A: 100X, B: X400.


3
2
2
YP
1
A

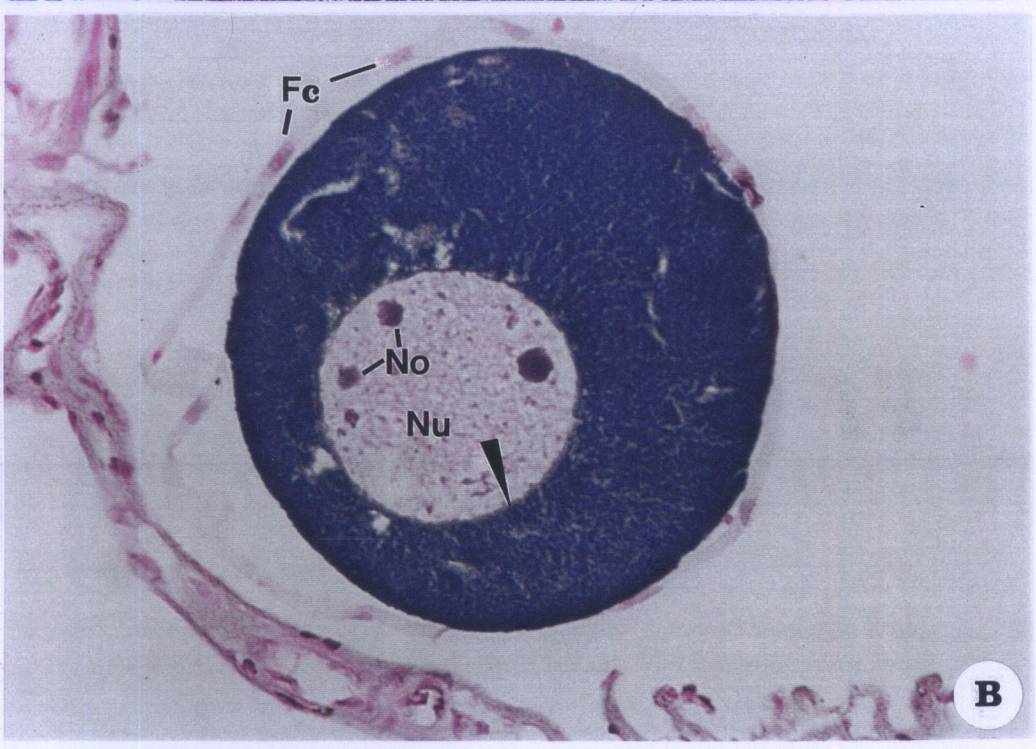

Fc
No
Nu
B

Figure 24 Light micrographs of H&E stained oocytes showing stage 2 and 3 oocytes.

A. The newly developed vacuoles (Va) are present on the periphery of stage 2. The mitonchondrial mass (mm) is indicated. Nu= nucleus, No= nucleoli, Fc= follicle cells. X400.

B. Early stage 3 oocyte showing wavy nuclear membrane (arrowhead). Nu= nucleus, No= nucleoli. X200.

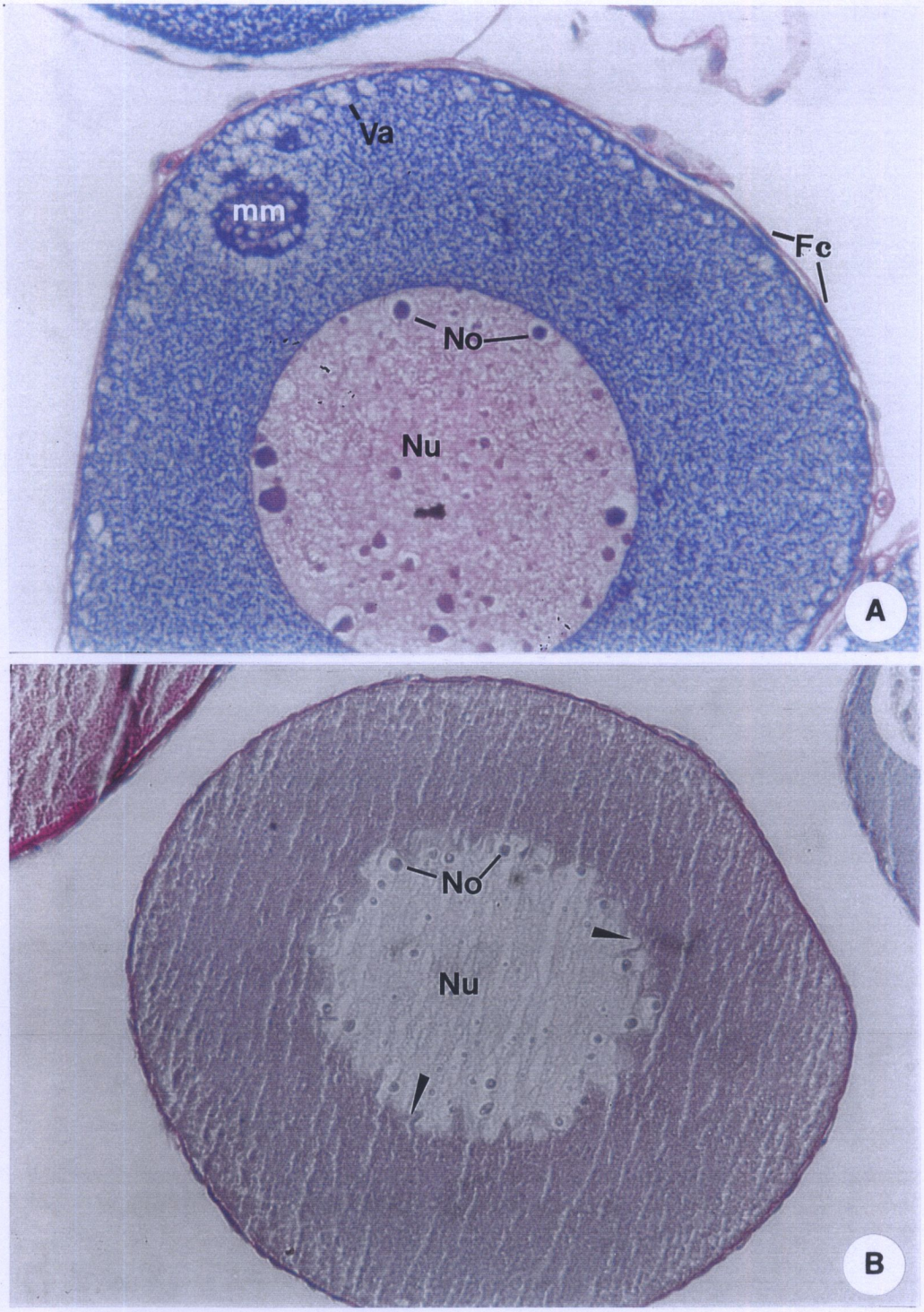

Va
mm
Fc
No
Nu
A
No
Nu
B

Figure 25 Light micrographs of H&E stained postvitellogenic (stage 6) oocyte.

A. Animal pole (AP) and vegetal pole (VP) are indicated. X40.

B. Highly sacculated nuclear membrane (arrowhead) and various size nuclei (No) are observed in stage 6 oocyte. X100.

C. High magnification of the animal pole (AP), pointing out the thick vitellogenic coat (VA), pigmented layer (PL), small yolk platelets (YP), and only one row of vacuoles (Va) on the periphery whereas two or three rows of vacuoles are present in the vegetal pole. VC= vitelline coat, Fc= follicle cell. X1000.

D. High magnification of the vegetal pole (VP), showing the absence of pigment layer and rather large yolk platelets (YP) in this pole. Va= vacuoles, VC= vitelline coat, Fc= follicle cell. X1000.

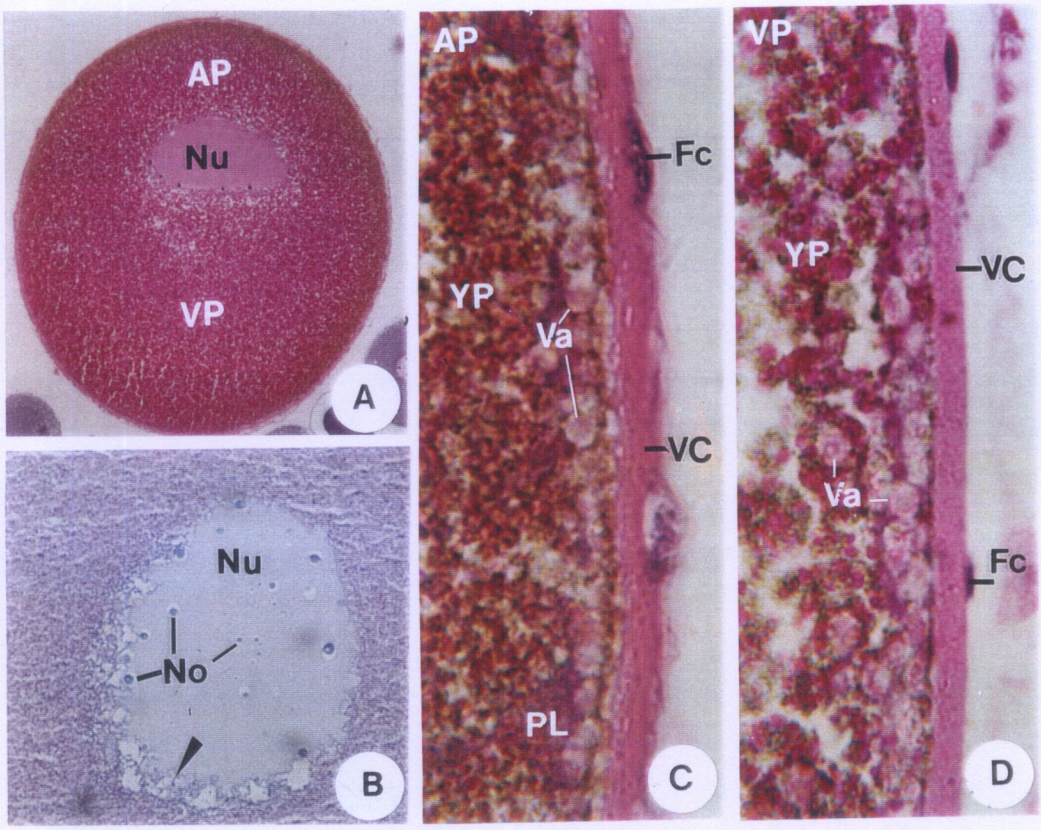

AP
Nu
VP
A
Nu
No
B
AP
Fc
YP
Va
VC
PL
C
VP
YP
VC
Va
Fc
D

Figure 26 Transmission Electron micrographs of small previtellogenic oocyte.

A. Previtellogenic oocyte illustrating the organization of the oocyte coat. SE,surface epithelium ; T, theca layer; FC, follicle cell; Mi, mitochodria. X10000.

B, C, D. Previtellogenic oocytes illustrating the presence of vacuoles (Va) associated with lysosome (Ly), bundles of fine filament (arrowheads), microvilli (Mv), mitochondria (Mi) and aggregation of lysosomes (arrow). B: X8000; C: X10000; D: X17000.

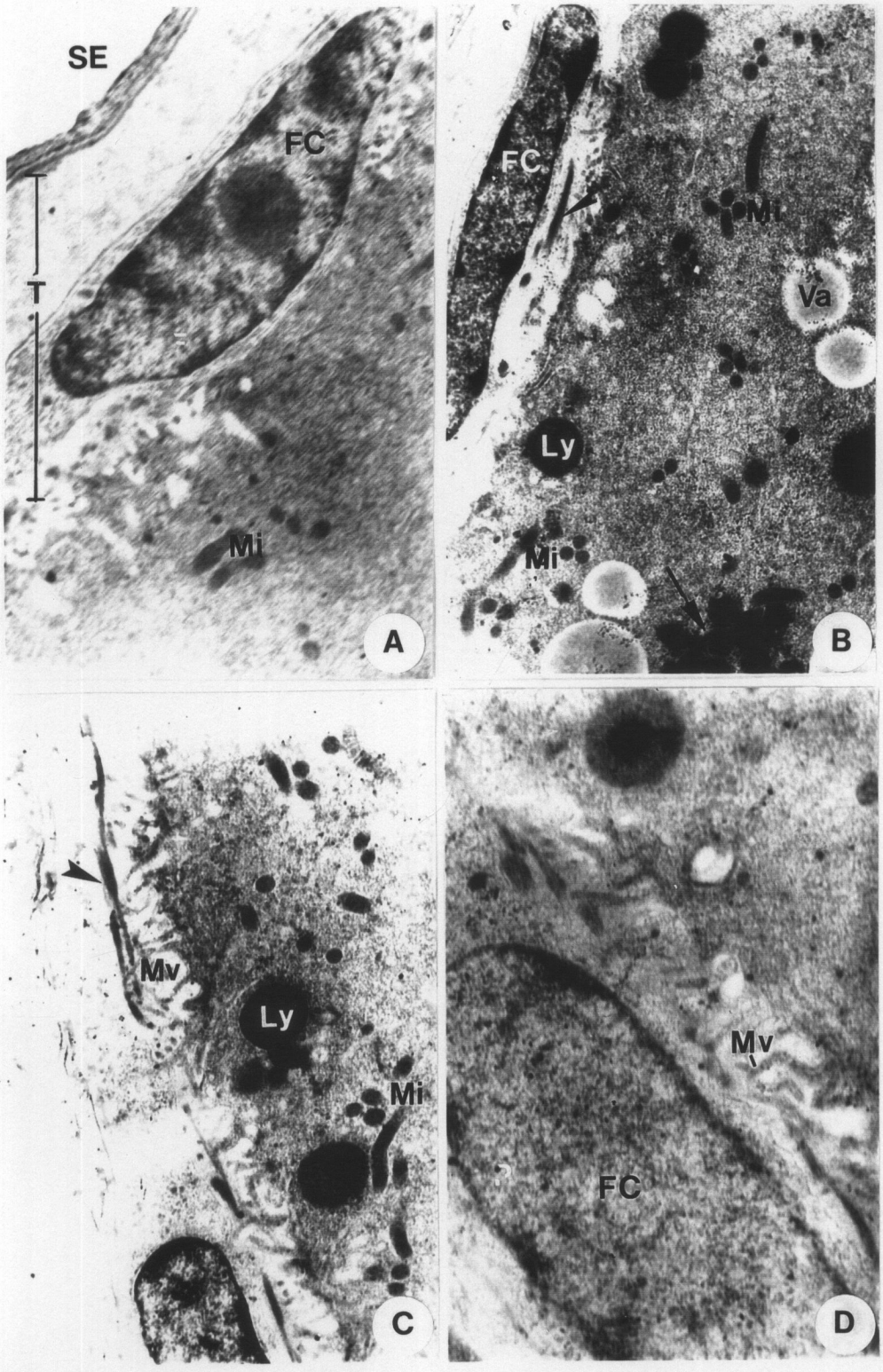

SE
FC
T
Mi
A
FC
Mi
Va
Ly
Mi
B
Mv
Ly
Mi
C
Mv
FC
D

Figure 27 Transmission Electron micrographs of stage 3 oocyte

A. Newly synthesized yolk (Yp) are maked in this stage. Va= vacuole; VE= vitelline envelope

B. Pinicytotic vesicles are investigated from the oocyte membrane (arrowhead). MV= microvilli

C. Crowded mitochondria (Mi) and pigment granules (arrow) are observed in this stage. YP= yolk platelete.

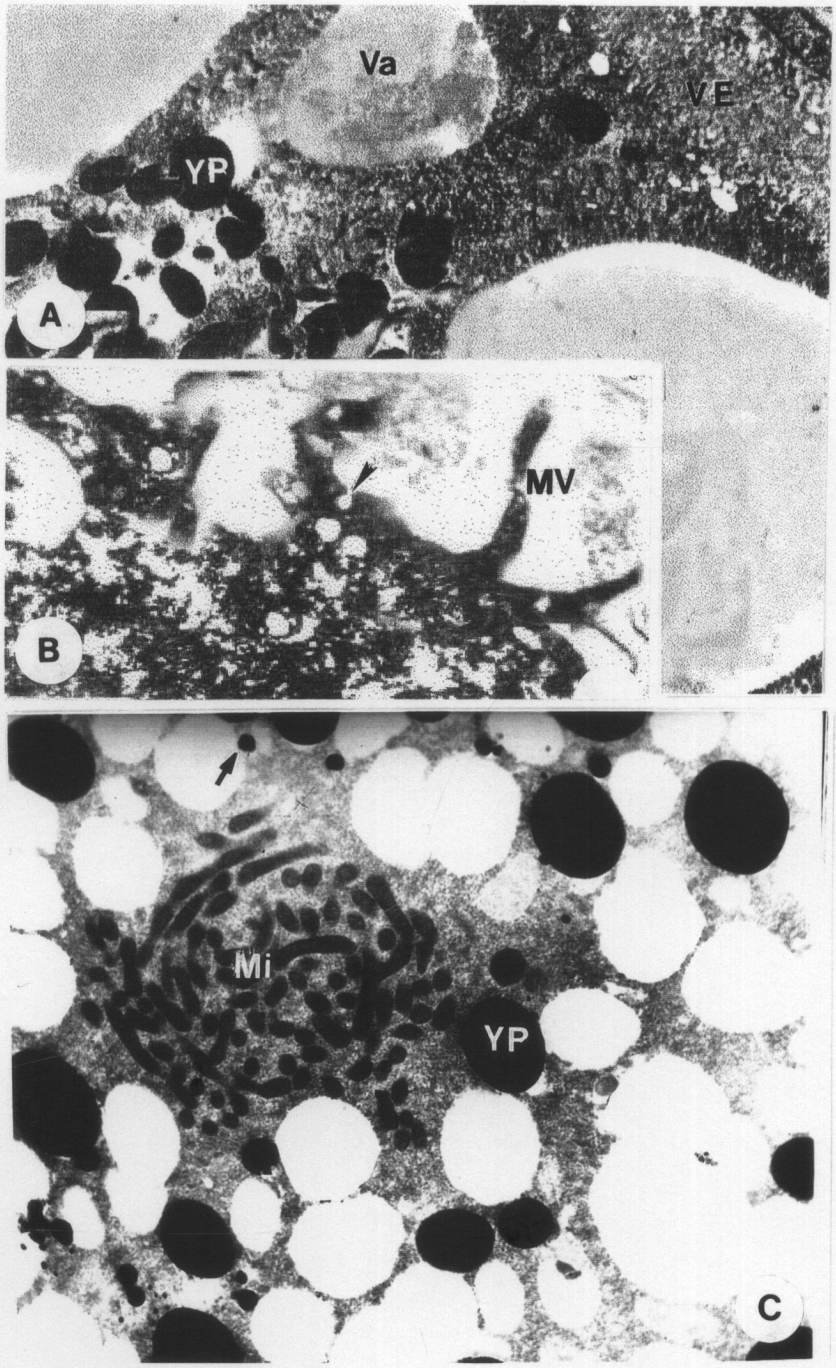

Va
VE
YP
A
MV
B
Mi
YP
C

Figure 28 Transmission Electron micrographs of postvitellogentic oocytes.

A. The mitochondria (Mi), Lipid droplets (Li), pigmented granules (P) and small yolk plateletes (YP) are indicated at animal pole. VE= vitelline envelope. X10000.

B. The pigmented granules (arowwhead) are also present in perinuclear cytoplasm. Lipid dropletes (Li) and yolk platelets(YP) are indicated. X3000.

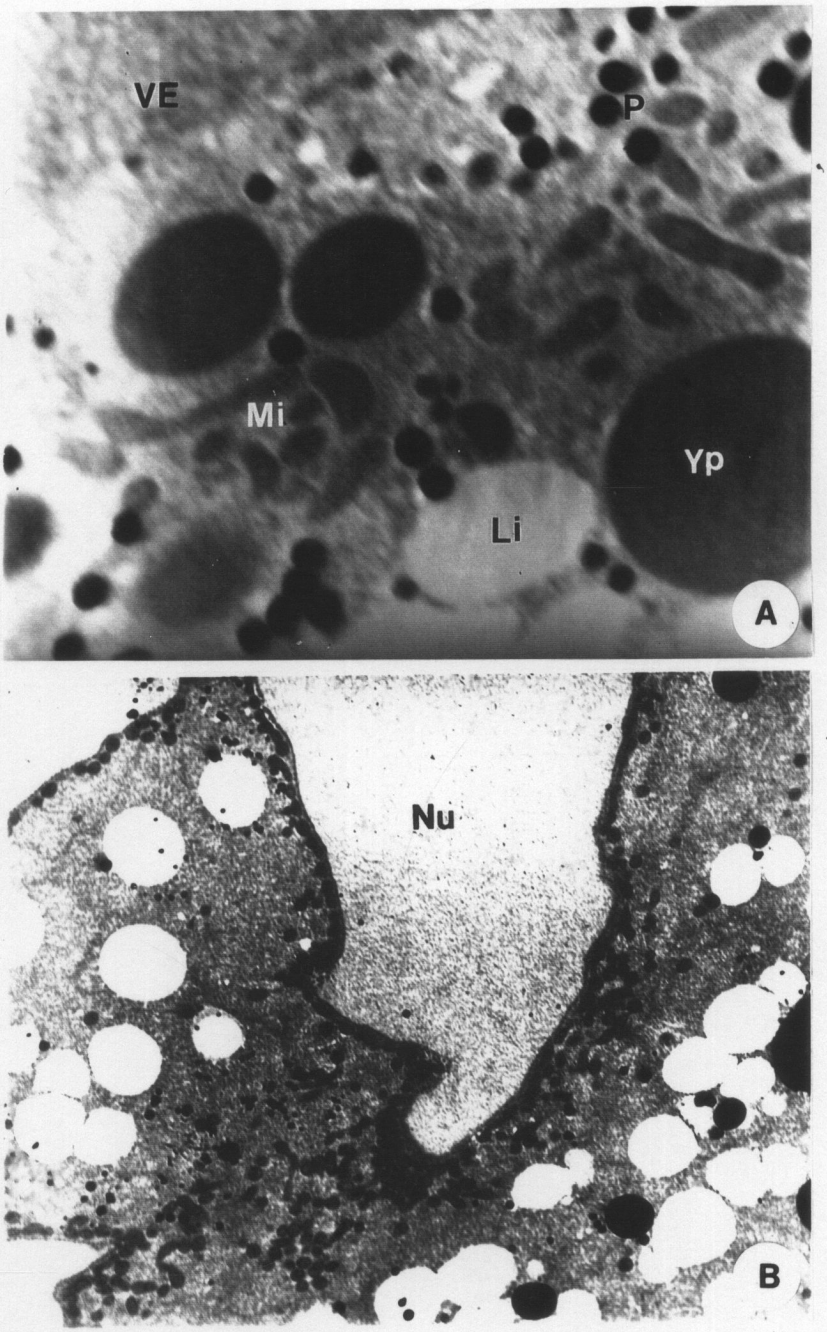

VE
P
Mi
Yp
Li
A
Nu
B

Figure 29 Transmission Electron micrographs of the animal pole of postvitellogenic oocyte.

A. The animal pole showing pigment granules (P), yolk plateletes (YP) the cortical granules (CG) which are close to the oocyte membrane. MV= microvilli; VE= vitelline envelope.

B. Fibrous components (F) are indicated in vitelline envelope.

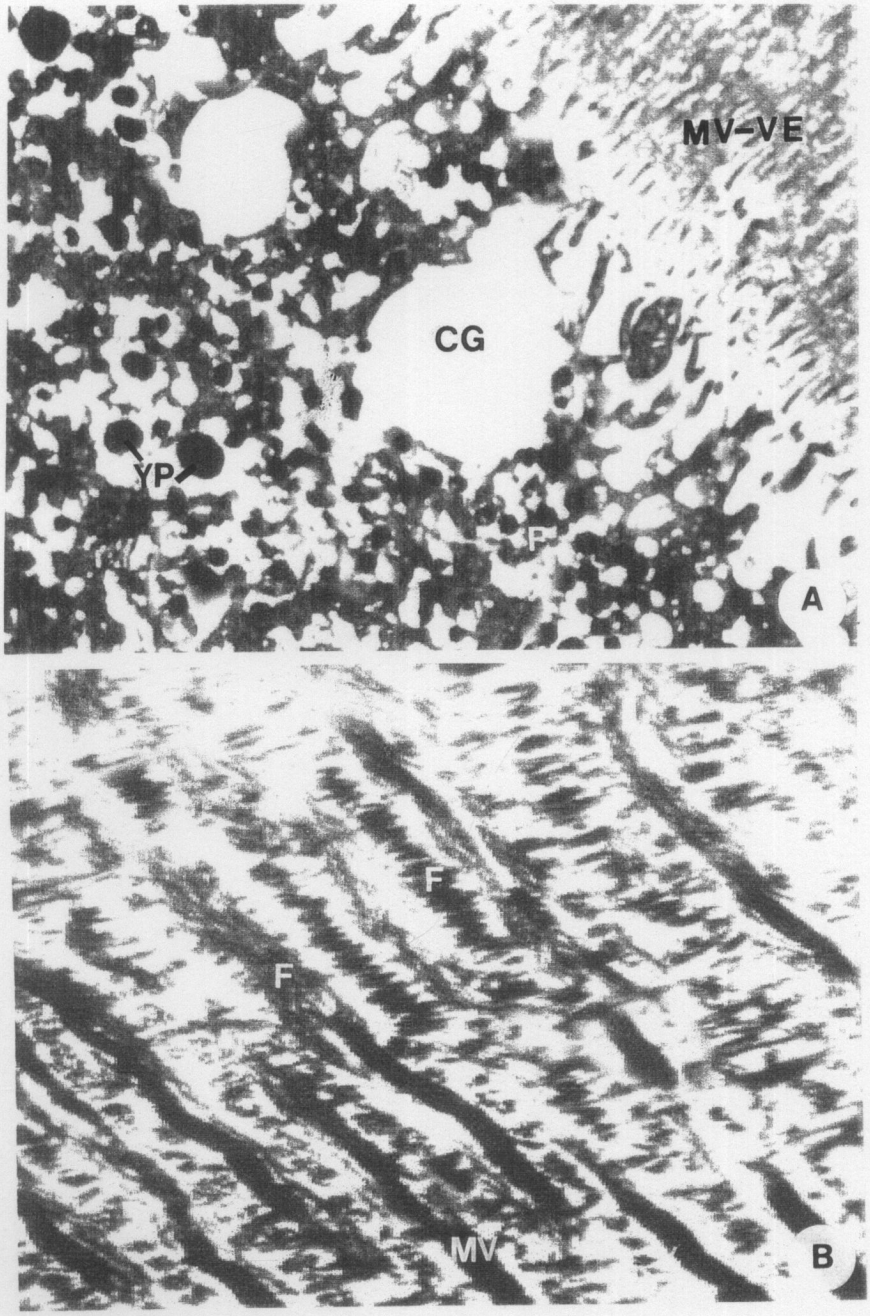

MV-VE
CG
YP
P
A
F
F
MV
B

Figure 30 Seasonal differences in the spermatogenetic activity and the proliferation of Leydig cells. a, the nest of primary spermatogonia; b, the nest of secondary spermatogonia; c, the nest of primary spermatocytes; d, the nest of secondary spermatocytes; e, the nest of spermatids; f, spermatozoa; g, Leydig cells.

A. The main spermatogenetic activity during cold season and hot season, the presence of cell nest and also some newly formed spermatozoa in the lumen. The Leydig cells are flattened and contain fine chromatin material. X200.

B. Massive spermatozoa were formed during the wet season. Leydig cells were abundant in the intertubular space. X400.

C. The seminiferous tubules in October (postbreeding season) showing relatively few cell nests most of which are in the spermatogonia stage and the maturing spermatids which anchoring to Sertoli cells; also the unshed free spermatozoa from the previous breeding month (August). X200.

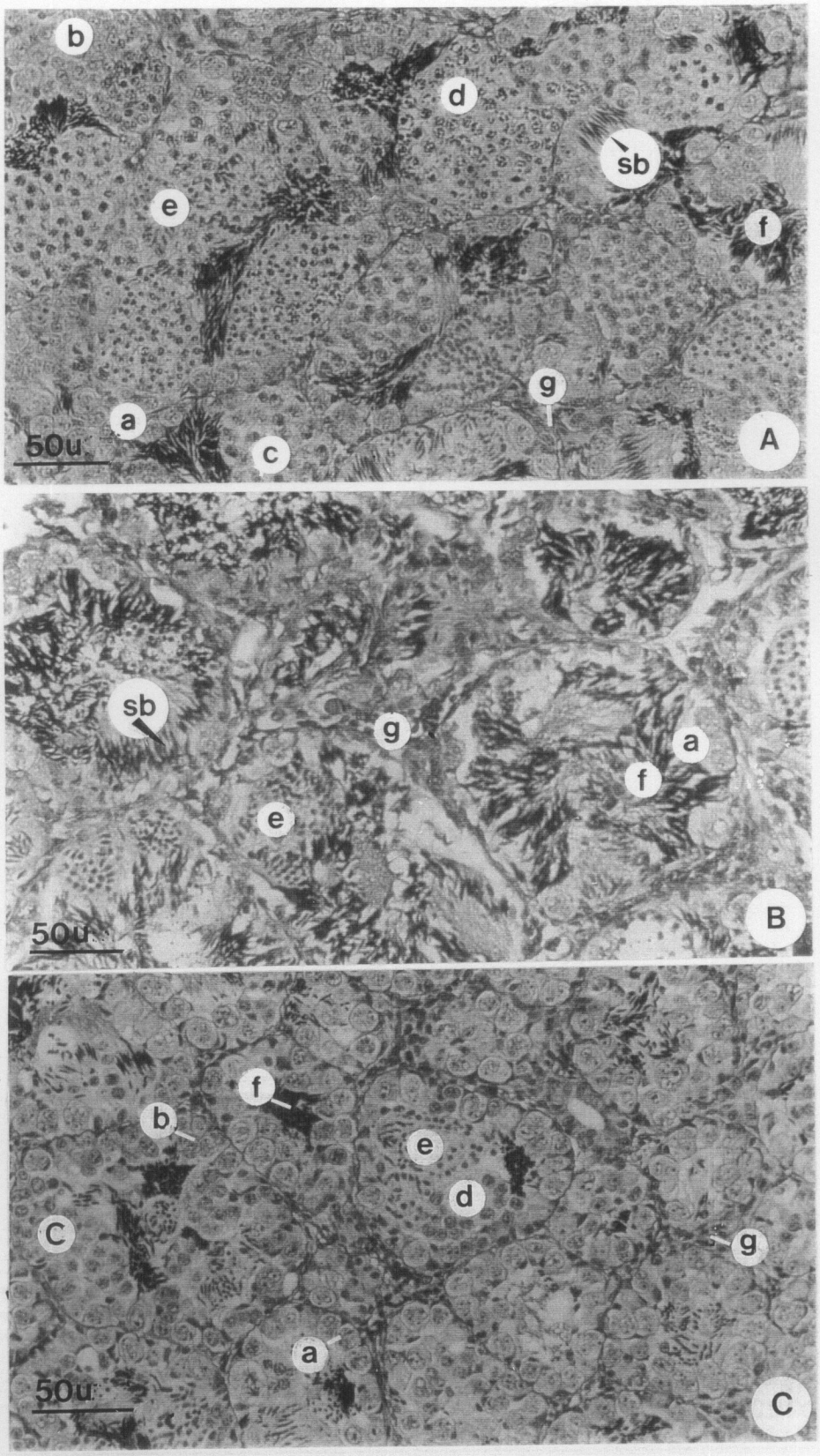

b
d
sb
e
f
g
a
c
50u
A
sb
g
a
f
e
50u
B
f
b
e
d
C
g
a
50u
C

Figure 31 Light micrographs of seminiferous tubules during the hot season and the wet season.

A. Seminiferous tubules showing clusters of sperm or sperm bundles (SB) which anchoring to Sertoli cells (SC). X1000.

B. Leydig cells (LC) surround the seminiferous tubules. X400.

FC= follicle cell

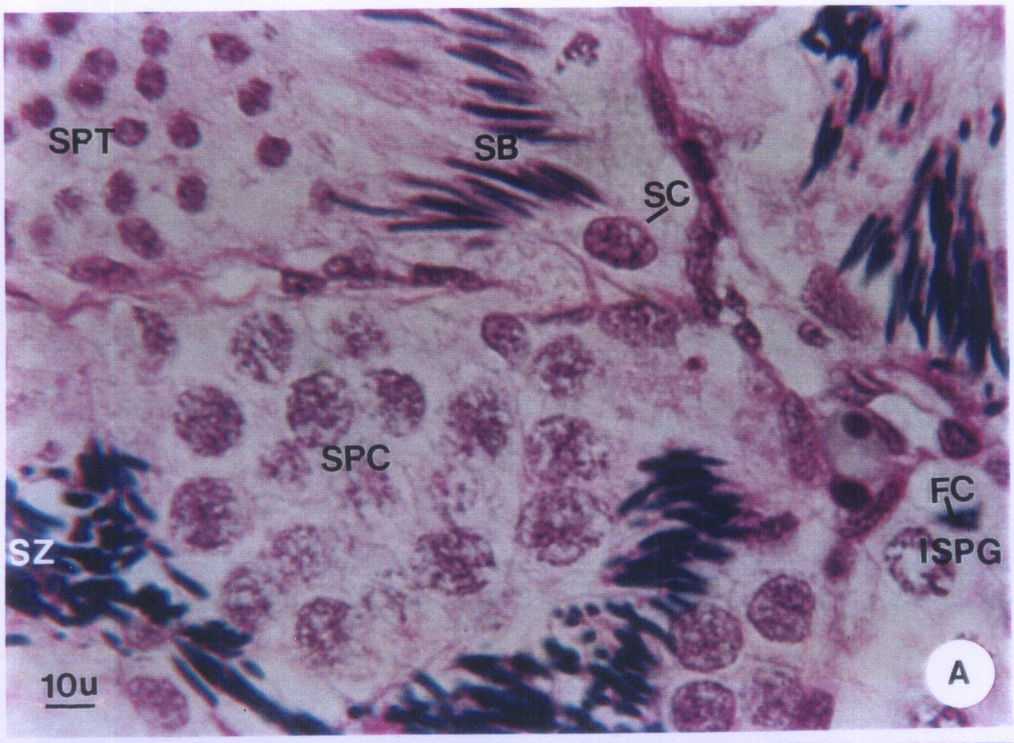
ISPG= primary spermatogonia

SPC= spermatocytes

SB= sperm bundles

SC= Sertoli cells

SZ= spermatozoa


SPT
SB
SC
SPC
FC
ISPG
SZ
10u
A

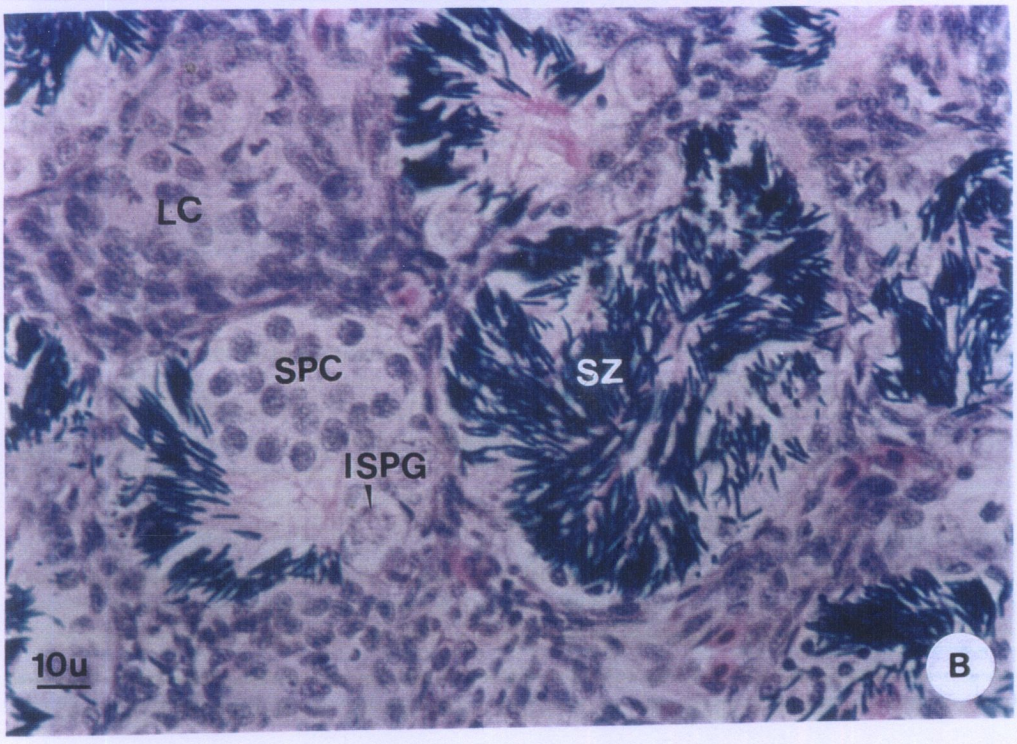

LC
SPC
SZ
ISPG
10u
B

Figure 32 Light micrographs showing seminiferous tubule with active spermatogenesis. A, B: The spermatogenetic activity cycle, showing all stages of germinative cells (the cold season). X400.

SPG = spermatogonia

SPC = spermatocytes

SPT = spermatids

SB = sperm bundles

SZ = spermatozoa

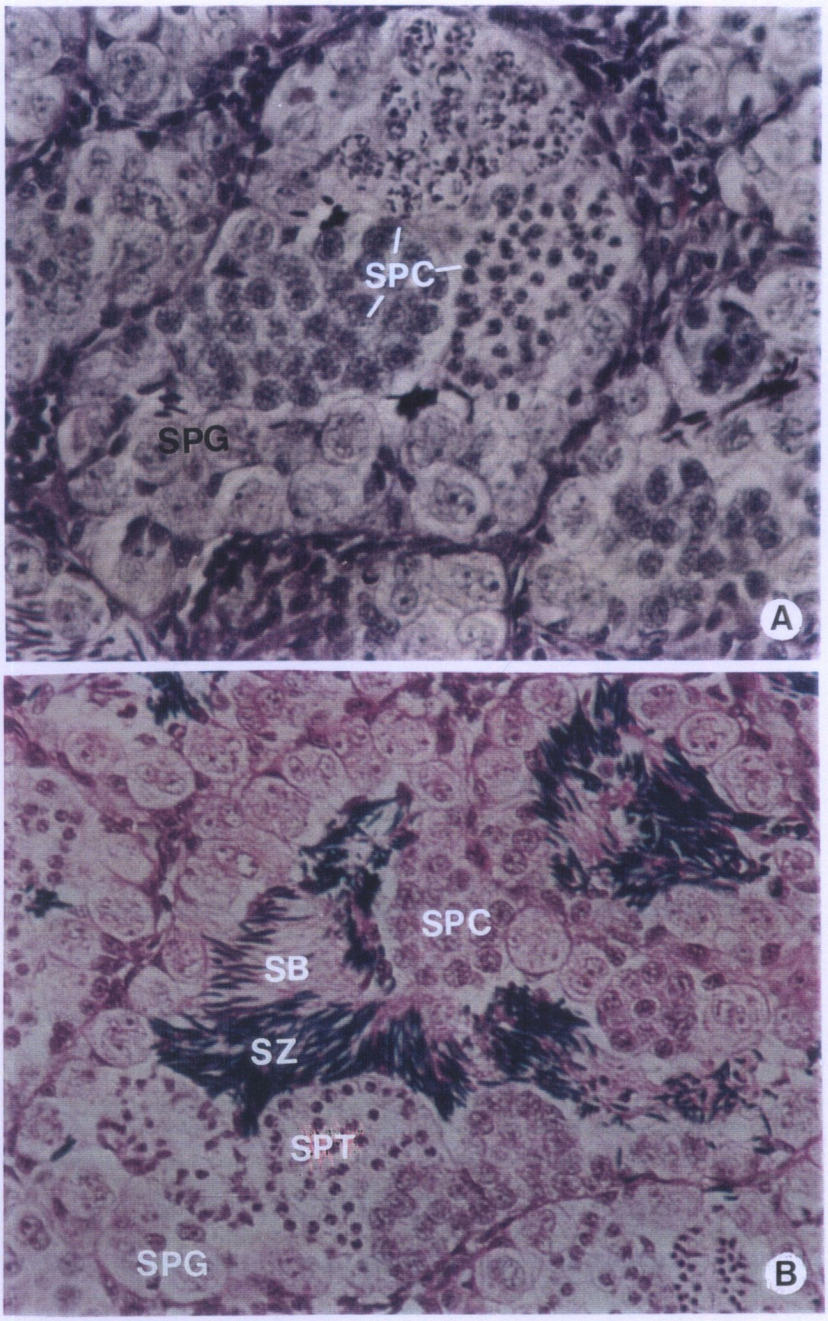

SPC
SPG
A
SPC
SB
SZ
SPT
SPG
B

Figure 33 Light micrographs showing seminiferous tubule with maximum levels of spermiogenesis and spermiation occurred during the end of the hot season (A) through the wet season (B), mainly in the middle of May to September. X400.

SPG = spermatogonia

SPC = spermatocytes

SPT = spermatids

SB = sperm bundles

SZ = spermatozoa

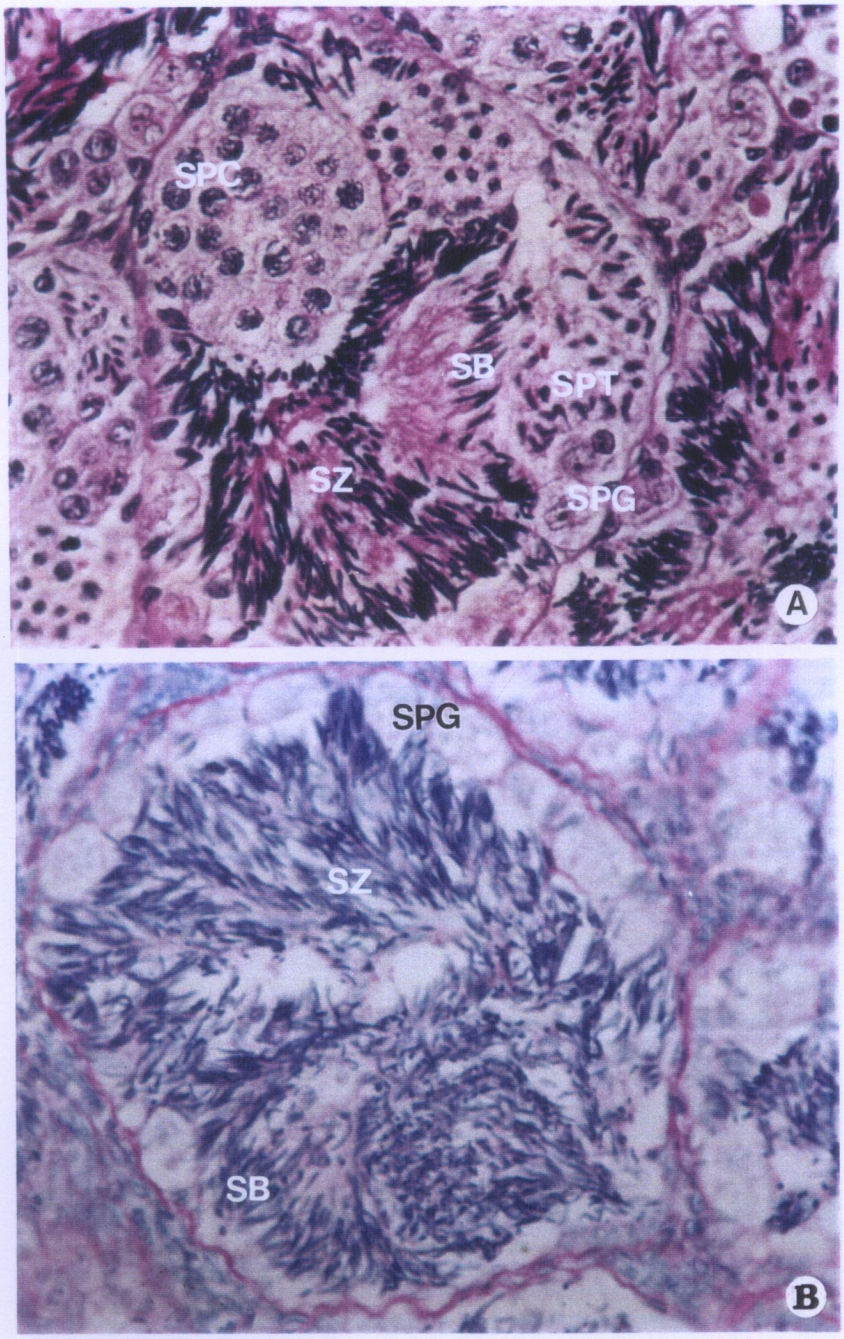

SPC
SB
SPT
SZ
SPG
A
SPG
SZ
SB
B

Figure 34 A, B: Light micrographs showing seminiferous tubule with relatively few cell nests (CN). X400.

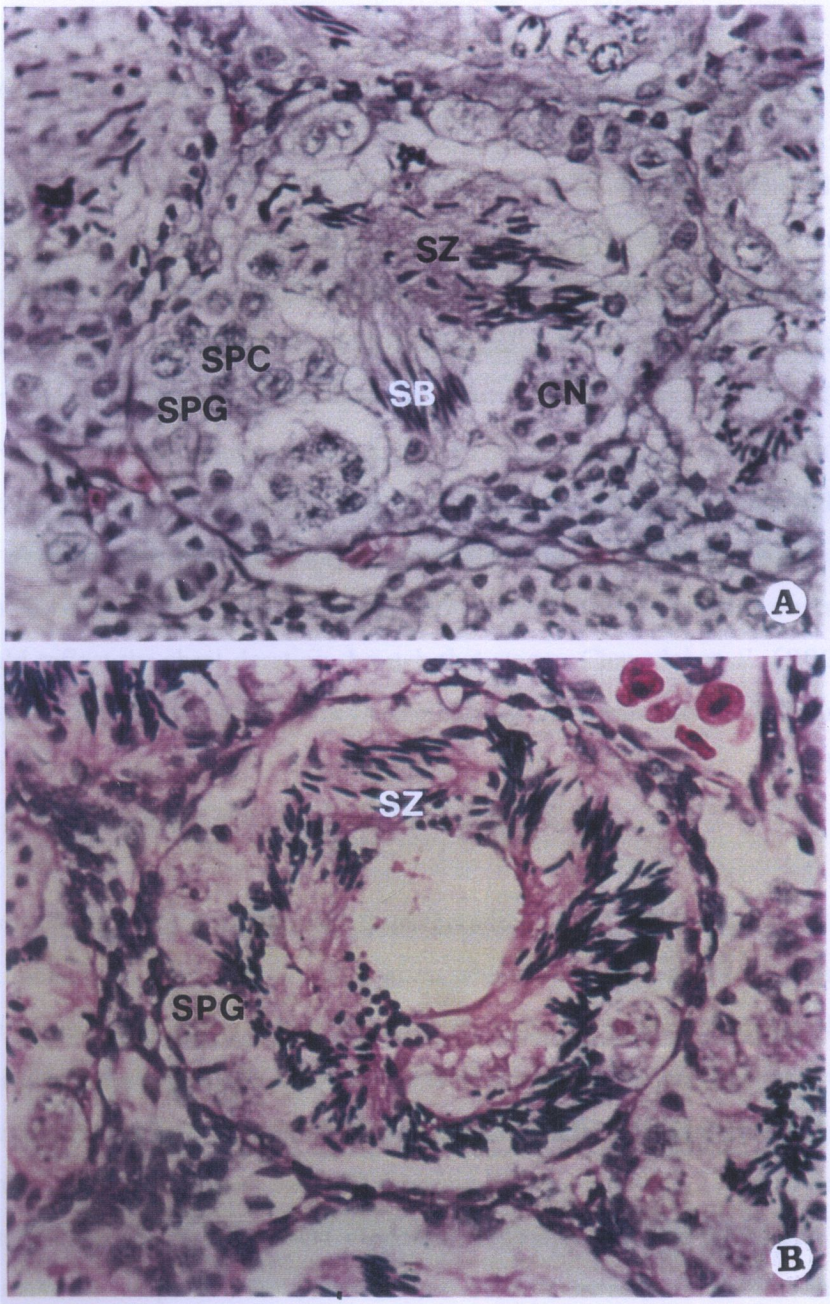
SPG = spermatogonia

SPC = spermatocytes

SPT = spermatids

SB = sperm bundles

SZ = spermatozoa


SZ
SPC
SPG
SB
CN
A
SZ
SPG
B

DISCUSSION

Gonadal Morphology

Morphology of Male Germ Cells

In comparison to *K. mediolineata* observed in the present study, the toad, *Bufo arenarum* could be divide male germ cells into 8 stages i.e. primary spermatogonium, secondary spermatogonium, spermatogocyte I, spermatogocyte II, three spermatid stages and spermatozoa (Houssay, 1954). Kalt (1976) and Callard *et al.* (1978) have studied testes of male *Xenopus laevis* and found 11 stages of germ cells. Rastogi *et al.* (1983) studied adult male *Rana esculenta* maintained at 18°C and also found 11 stages of spermatogenic cells. The relative durations of various cell stages could be inferred from the light microscopic observations. It is generally assumed that at any instant cells with longer duration should be present in more numerous number while the scarce cells observed in each section should reflect the short duration through that stage. Chavadej *et al.* (2000) reported that spermatogonia, pachytene spermatocytes, middle stage spermatids, and differentiating spermatozoa had long duration while secondary spermatocytes, diplotene and diakinesis-metaphase spermatocytes had comparatively short durations.

In transmission electron microscopy observations, most notable were the complete euchromatic material in primary spermatogonium which transforms into small blocks of heterochromatin that were partly attached on the inner surface of the nuclear envelope and partly scatterred throughout the nucleus in secondary spermatogonium. In spermatocyte, the chromatin turned into large discrete clumps or bundles which individual chromatin fiber became increasingly condensed as the cells progress to the next stage. The maximum condensation of chromatin bundles were observed in the zygotene and pachytene stages where synaptonemal complexs were also detected. Due to the reduction of the nuclear size in diplotene spermatocytes, the completely condensed chromatin blocks were packed closer. Secondary spermatocytes showed highly condensed chromatin in the forms of X and Y figures. The nucleoli which are quite prominent in primary and secondary spermatogonia begin to disappear in early spermatocytes.

The cytoplasmic volume as well as cell organelles also change during spermatogenesis. There was gradually reduction of the cell size from 14-16 um in primary spermatogonia to about 8-10 um in diplotene spermatocytes. Reed and Stanley (1972) reported similar changes as in the early stages of male germ cells in *Xenopus laevis.* During spermiogenesis of *K. mediolineata* the nucleus of early spermatid was still round but the size was reduced to about 5 um. The nucleus started to appear oval in late spermatid and changed to elongated shape in spermatozoa. The heterochromatin blocks as seen in spermatocytes dissappeared and were replaced by evenly dispersed chromatin granules. During chromatin condensation, the chromatin granules within the herteochromatin blocks continually increased in size, However, the packing of these granules started from over one-haft of the nucleus in early spermatid to fully uniform but still well separated from one another in round spermatid and tightly packed in late spermatids and spermatozoa. This tight packing was also assisted by the reduction of the nucleus size and the change in shape to ellipsoidal form. Zirkin (1971) had classified spermiogenesis in *Rana pipiens* into 5 stages: early spermatid, 3 stages of middle spermatids with round to cigarlike nucleus, and spermatozoa. The packing of chromatin granules (fibers) was quite similar in *R. pipiens* and *K. mediolineata*. Such characteristics chromatin packing were also observed in other vertebrate species and were easily noticed in amphibians as point out in the study of toads. Burgos and Fawcett (1956); Zirkin (1971) putatively explained that the replacment of histones by protamines, another basic sperm proteins, was instrumental increasing the size of the chromatin granules and packing them close together. Acrosome of *K. mediolineata* spermatozoa started appearing in round spermatid stage. This organelle derived from the Golgi membrane which began as flattened sac covering the anterior half of the nucleus. Some organelles, particularly rough endoplasmic reticulum disappeared while mitochondria still remained. The cytoplasm was gradually extruded towards the posterior and broken down into cytoplasmic debris. Eventhough most of the remaining cytoplasm was eventually discarded, the cytoplasm remaining at the neck area is still quite substantial when compared to mammalian spermatozoa (Reed and Stanley, 1972; Zirkin, 1971). Another departure from the mammalian scheme of spermatogenesis was the development of clones of germ cells inside germinal cyst or cell nest. In mammalian species the daughter cells derived from single spermatogonium and retained the cytoplasmic syncytium only up to spermatocyte stages while those in amphibians, such as *K. mediolineata* in the present study, these cells were still surrounded by definite layer of follicle cells. This encasement persisted upto the spermatid stages. The cyst might rupture when the spermatozoal stage was attained in which

late spermatids and young spermatozoa had their heads embedded within the cytoplasm of Sertoli cells. It was possible that follicular cells might also evolve, concurrently with the process of spermatogenesis and spermiogenesis, into Sertoli cells at the final stage of maturation. Virtually, this proposition was supported by others' studies, such as in newt and urodele amphibians (Tso and Lofts, 1977).

Seasonal Differences in the Morphological Appearance of Leydig and Sertoli Cells

Distinct seasonal differences in the morphological appearance of Leydig and Sertoli cells were apparent when observed at different periods of the median-striped burrowing frog reproductive gonadal cycle. Leydig cells of *K. mediolineata* testis at the time of full spermatogenesis were structurally well differentiaed and numerous, particulary during the breeding time (in the wet season). Their cytoplasm possessed numerous dense granules, lipid droplets and mitochondria with tubulovesicular cristae. These are characteristics of secreting steriodogenesis tissues (Christensen and Gillim, 1969; Saidapur and Nadkarni, 1975).

With the progression of spermatogenesis, Sertoli cells hypertrophy, accquire smooth endoplasmic reticulum, rough endoplasmic reticulum, spherical mitochondria with tubular cristae,lipid droplets (Lofts et al., 1972). Similar morphological changes have been found in *K. mediolineata.* Especially, maximum levels of spermiogenesis and spermiation occurred from the hot season through the wet season, mainly in the middle of April to Augustwhen clusters of sperm attached to Sertoli cells, or free spermatozoa were observed in the lumen. At this stage in Sertoli cell of *R. temporaria* resembles that of the mammalian testis, and has all the characteristics of a steroid-producing tissue (Brokelmann, 1964).

Reproductive Testicular Cycle

Three major environment factors have been implicated in the regulation of the amphibian breeding cycle: rainfall, photoperiod, and temperature (Lofts, 1974). Rastogi *et al.* (1978) studied various environmental influences which could alter the characteristics of internal morphology of the testis in *Rana esculenta* and found that these influences consisted of rainfall, temperature, and photoperiod. These factors caused cyclical internal changes of the testis. Loumbourdis *et al.* (1991) reported that *Rana ridibunda* showed significantly

increase in number of spermatids during breeding season around April to June while in winter the number of spermatocytes was decrease and reached minimum in the coldest month. In the current study area where the wet and dry seasons are quite distinct. *K. mediolineata* showed responses to seasonal differences in reproductive capacity. During the wet season (May-August), the seminiferous tubules produced spermatid and spermatozoa in full steam. In postbreeding period (September-October), these tubules consisted of few cell nests. During the cold season such tubules had rapid increase in number of cell nest. There were numerous spermatocytes and spermatogonia in preparation for subsequent development. Maximum levels of spermiogenesis and spermiation ocurred during the hot season through the wet season. Such cyclic change might depend on the availability of gonadotropin as has been demonstrated in *R. temporaria* that exogenous administration of these hormones or the elevation of environmental temperature could stimulate recrudescence of spermatogenetic activity in the seasonally quiescent males (Van Oordt, 1960). Jorgensen *et al.* (1979) studied in *Bufo bufo* and reported that this toad species showed similar cycles to seasonal differences. Lofts (1964) studied the green frog, *R. esculenta*, and found the same result as those such two species. Another evidence supporting the response of testis to seasonal differences is the gonadosomatic index (GSI). Kao *et al.* (1993) showed that GSI in *R. rugulosa* exhibited the rising phase in hibernation and prebreeding season while the maximum GSI were observed in the breeding period. On the other hand, GSI decreased in late breeding period and remained at low level in postbreeding period. In *K. mediolineata*, the changes of GSI exhibited slightly increase in GSI and it was interesting that the active spermiogenesis and spermiation occured even before the wet season in which during hot season. Furthermore, declining of spermatogenesis commenceed before the end of wet season which was around October.

Testicular Activity

Amphibians inhabiting tropical areas where climatic condition do not show appreciable fluctuations have often developed continuous type of spermatogenetic cycle. Whereas other species in temperate regions have developed discontinuous type or potentially continuous type of spermatogenetic cycle (Basu and Nandi, 1965; Lofts, 1974). In species with continuous type of spermatogenetic cycle, the spermatozoa are normally produced throughout the year and the testes always contain spermatic cell nests as well as complete spectrum of spermatogenetic stages (Sun,1979; Saidapur and Kanamadi, 1982; Saidapur,

1989). In contrast, the spermatogenetic activity of species living in the temperate regions is restricted to late spring and summer. Such discontinuous production of spermatozoa has been attributed, at least in part, to the seasonal lowering of environmental temperatures and accompanying decline of gonadotropin output (Lofts, 1974). Mondal and Basu (1960); Lofts (1974); Saidapur (1989) proposed that the spermatogenetic cycle of species which have free sperm in the seminiferous tubules belongs to the continuous type. In other words, the classification of spermatogenetic cycles of anuran species belonging to continuous or discontinuous types depends on the presence or absence of free sperm in the seminiferous tubules. As observed in the present study, *K. mediolineata* had free sperm in the seminiferous tubules the entire year. According to the categorizations by Lofts (1974) ; Rastoki *et al.*1976; Saidapur (1989), the spermatogenetic cycle of *K. mediolineata,* thus, belongs to the continuous type. However, the observation of the numbers of free sperm showed that it was higher in the breeding period than any of the other periods. Such observations revealed that although *K. mediolineata* belongs to the continuous type of spermatogenetic cycle, it has distinct breeding time. However, it is inappropriate to determine the nature of the reproductive gonadal cycle of frogs by depending solely on the presence of free sperm in the seminiferous tubules. From present study, it might propose that the continuous type of spermatogenetic cycle of anurans could be devided into constantly continuous type, e.g., *R. cyanophlyctis* (Saidapur and Kanamadi, 1982), and the fluctuating continuous type, e.g., *K. mediolineata*, in the present study.

Association of Environmental Factors with Reproductive Testicular Cycle

Effects of environmental cues on male anuran reproductive activities have been studied (Lofts, 1974; Rastogi *et al.*, 1976, 1978 ; Saidapur, 1989; Delgado *et al.* 1989). It was reported in most subtropical and tropical species that rainfall is the most important factor associated with initiation of breeding activity since temperature changes little with seasons in such area (Lofts, 1974). On the other hand, temperature is the primary factor in temperate species which exhibiting cyclic reproductive pattern with season and plays an important role in synchronizing different phases of the seasonal testicular cycle in several anurans (Rastogi *et al*, 1976,1978; Iela *et al.*, 1980; Pancak and Taylor, 1983; Pierantoni *et al.*, 1985). In the area of Nongteng-Chakkarat National Reserved Forest, *K. mediolineata*, the tropical anuran species, revealed the apparent correlation between rainfall and testicular activity.

Morphology of Oocytes

The criteria for dividing oocytes into various stages are mainly based on the size, the amount and the distribution of yolk and pigment (Kemp, 1953; Grant, 1953; Wartenberg , 1962) and the morphology of chromosome (Duryee, 1950). In the present study, several criteria were used for characterizing in staging of oocytes of *K. mediolineata.* These included the size, color, as well as the internal morphology.

The outer coat of oocytes is composed of surface epithelium which comprising of connective tissue layer and follicle cell layer as well as layer of vitelline fibers aranged in three directions (called the vitelline envelope or coat). This feature is common in the most species of anurans. The formation of the vitelline envelope in *K. mediolineata* could be first detection as isolated bundles of fine filaments in the stage 2 oocyte similar to those found in oocytes of *Xenopus laevis* (Dumont, 1972). In *X. laevis*, the microvilli extending from the oocyte surface gradually increased in number and length particularly in stage 3 and 4 oocytes (Wallace and Selman, 1990. These changes of microvilli are similar to those observed in the oocyte of *K. mediolineata* in the present study. In addition, the present study revealed that the full thickness of vitelline envelope was observed in the stage 5 oocyte. The increased number and the length of microvilli might be need to increase the surface area of oocyte during development. Since amphibian oocytes must store nutrient materials in the form of yolk platelets, they need relatively large surface area for the uptake of the nutritive substances which are necessary for the yolk formation. Corresponding to this demand, during stage 3 and 4 oocytes of *K. mediolineata*, there were extensive pinocytotic activities which might reflect the mechanism whereby materials enter the cytoplasm through the endocytotic pathway as reported in *X. laevis* (Wallace and Selman, 1990). Pan *et al.* (1969) reported that vitellogenin or yolk protein is the precursor of yolk platelets during vitellogenesis. The term vitellogenesis means the process of synthesizing yolk platelets and includes vitellogenin formation from liver (Follett and Redshaw, 1967). Follett and Redshaw (1967) used serum lipophosphoprotein (SLPP) to substitute for vitellogenin. SLPP is transported *via* blood circulation to the ovaries where it is taken up by the oocytes under the influence of Gonadotrophic hormones. After transport into the oocytes, SLPP is dissociated into two components, phosvitin and lipovitellin, and these are finally reconstituted to form yolk platelets. The present study had demonstrated that the yolk formation began in

stage 3 as in *X. laevis* (Dumont, 1972). Perhaps this occurs through the increased receptor-mediated endocytosis (Dumont, 1978).

Mitochondrial clusters observed in vitellogenic oocytes in this study were commonly observed in many anurans (Ward and Ward, 1968). This might be due to the need for large energy supply for the synthesis of new macromolecules and for the assembly of various structures in the oocytes. The increase of mitochondria numbers during vitellogenesis might be due to their rapid proliferation as it had been suggested that out of the total of 16-17 rounds of mitochondrial DNA replication during oogenesis, 12 rounds took place before the onset of vitellogenesis (Brachet and Alexandre, 1986). During development of oocyte of *K. mediolineata* , the nuclear envelope changed from having smooth contour in the earlier stages to being more highly folded in later stage . This change might be in response to the need for a large surface area for the transport of various classes of RNA to cytoplasm and, subsequently the reverse transport of proteins into nucleus across the nuclear membrane *via* nuclear pores (Brachet and Alexandre, 1986).

Seasonal Differences of the Ovary and Climate

One of the most remarkable characteristics of amphibians is the change of ovarian cyclicity in correlation with the variation in environmental or seasonal condition (Kanamadi and Saidapur, 1982), especially the seasonal climatic cycle of temperature and precipitation. In tropical countries where there are pronounced wet and dry seasons, the breeding and non-breeding periods are clearly separated. In India (in Dharwar city, 400 km. northwest of Bangaore) the main breeding season of frogs coincides with the peak of monsoon rain during June to August (Jorgensen *et al.*, 1979). The climate in the present study is also pronounced wet and dry seasons and found that breeding time of *K. mediolineata* coincides with the period of monsoon rain that generally extends from May to October. The appearance of stage 1 and 2 oocytes or small previtellogenic oocytes throughout the year as in other anurans. Sklavounou and Loumbourdis (1990) suggested that this frog species there was always reserved pool of oocytes. This study also revealed that the degenerated (or atretic) oocytes also appeared all year round and increased from October to February which correlateed with the decline of Stage 6 oocytes. This suggests that atresia during such period was due to the degeneration of the stage 6 oocytes which could be due to the lower level of gonadotropins (Hoque and Saidapur, 1994; Pramoda and Saidapur, 1984).

Recruitment of oocytes to vitellogenic growth commences in the hot season (March) throught the wet season. In the present study, the hypertrophy of the follicle cells commences in the hot season. They developed a prominent endoplasmic reticulum, mitochondria become more numerous, suggests increased activity during the prespawning period. Tests for steroid dehydrogenase activity have confirmed the ultrastuctural evidence of a steroidogenic activity (Saidapur and Nadkarni, 1975).

Accordingly, frogs with ovaries in postvitellogenic (stage 6) oocytes can be observed during May to December. However, postvitellogenic oocytes in October, November and December, possibly remnants of previous ovarian cycle. Moreover, the presence of stage 6 oocytes during May to August was correlated with the large amount of spermatozoa in the testis in male frogs. Thus, the period from the late May to August is referred as breeding period. The high incidence of such postvitellogenic oocytes coincides with the rainfall, However, the amplexing occurred two times in the months of high rainfall (in May and August) associated with high soil moisture and surplus water that form the temporary pond for amplexing. In the present study, the mating call was first observed on February 24th 2000 when the moderated rainfall at 13.7 mm but there were not any temporary pond for amplexing . It is observed that none of postvitellogenic oocytes occuring in ovaries within such month. On May 21th 2000, there was heavy rainfall (88.2 mm.) in which the mating call and amplexing were first observed at the temporary breeding pond and the tadpoles appeared in June. Such breeding behavior can be observed once again in the late August which very heavy rainfall. The tadpoles appeared in September and disappeared in the middle of October. The population of *K. mediolineata* may, therefore, be characterized as opportunistic breeders in which females with complements of full grown eggs breed in response to the establishment of suitable environmental conditions (Jorgensen, 1984). Lofts (1984) proposed that rainfall seem to have no any significant effects on amphibian gametogenetic activity but tended to influence the timing of reproduction in many anuran species by triggering migration, breeding and oviposition in the already sexually ripe individuals. This is particulary common in xeric species where breeding take place in temporary sites formed by sudden rains. Breeding in such forms is often explosive within a single night. The onset of the wet season may, thus, act as the trigger of explosive breeding activity within population of *K. mediolineata*. Recruitment and growth of a complemnet of vitellogenic oocytes could be found in *K. mediolineata* depend on the rain in summer storm

and the monsoon rain. Correspondingly in maximum levels of spermiogenesis and spermiation occured from the hot season to the wet season.

CONCLUSIONS

Male germ cells in the testes of adult *K. mediolineata*, endemic anuran species of Thailand, can be classified into twelve stages based on the nuclear characteristics as studied by light and electron microscopy. Primary spermatogonium that shows large euchromatic nuclei with prominent nucleoli. Secondary spermatogonium tends to show small heterochromatic blocks along the inner surface of the nuclear envelope and scatterred throughout cytoplasm. Spermatocytes comprise of five stages including leptotene, zygotene, pachytene, diplotene, and metaphase 1 spermatocytes. Chromatin condensation occures from leptotene stage to the highly condensed blocks in diplotene stage. Nucleoli are not detected in any stages but zygotene stage has synaptonemal complex. Secondary spermatocytes has heterochromatin blocks attaching to the nuclear envelope resembing clock-face pattern in light microscope, and X or Y figures in electron microscope. Spermatids comprise three stages. The early stage has partial chromatin condensation over one-half of the nucleus. The middle stage shows condensation of chromatin throughout nucleus which ovoid shape and acrosome also forms in this stage. The late spermatid exhibits completely chromatin condensation in otherwise ellipssoidal nucleus, also cytoplasm becomes highly vacuolized and begins to degenerate. The last stage of germ cells is spermatozoa in which head is elongate and highly condensed chromatin. Mitochondria in this stage are relatively short and not arranged into helical pattern like in mammalian spermatozoa. As in other anamniotes, spermatogenesis is of the cystic type, i.e., germ cell proliferation occures in a germinal cyst or cell nest or follicle. The cells present in cell nest are in the same stage of development and are derived from single spermatogonium. Each primary spermatogonium is completely enclosed by the follicle cells which later mature into secretory Sertoli cells. Leydig cells are found between seminiferous tubules.

Leydig cells of *K. mediolineata* testis at the time of full spermatogenesis were structurally well differentiaed, round in outline and numerous, particulary during the breeding time (in the wet season). Soon after the breeding, in October-November, the Leydig cells become flattened or shrunken. With the progression of spermatogenesis during the hot season and the wet season, Sertoli cells hypertrophy, accquire smooth endoplasmic reticulum, rough endoplasmic reticulum, spherical mitochondria with tubular cristae, lipid droplets.

K. mediolineata showed reproductive testicular cycle in response to seasonal differences. During the wet season (May-August), the semoniferous tubules produced spermatids and spermatozoa in large number. In postbreeding period (September-October), these tubules consisted of few cell nests. In the cold season such tubules had rapid increase in number of cell nest. There were numerous spermatocytes and spermatogonia in preparation for subsequent development. Maximum levels of spermiogenesis and spermiation ocurred during the hot season through the wet season. It was concluding that the active spermiogenesis and spermiation occured even before the wet season in which during hot season.

The spermatogenetic activity of *K. mediolineata* belongs to the continuous type. In the area of Nongteng-Chakkarat National Reserved Forest, *K. mediolineata*, the tropical anuran species , revealed the apparent correlations existing between rainfall and testicular activity.

The oocyte of *K. mediolineata* can be classified into 6 stages based on size, external and internal appearance. Stage 1 oocyte has translucent cytoplasm with 50-300 um in diameter It contains large number of mitochondria, lipid droplets and round nucleus. Stage 2 oocyte has opaque cytoplasm, ranging in size from 300-450 um. Mitochondria and lipid droplets are increased in number and dispersed throughout the cytoplasm. Vitelline coat and vacuoles or cortical granules appear in this stage. Some mitochondria consist of rectangular crystalline structures. Stage 3 oocyte is 450-600 um in diameter with intensely white. Yolk platelets and pigmented granules begin to form in this stage. Stage 4 oocyte is brown in appearance with 600-1000 um in diameter. Cortical granules, pinocytotic vesicle, and yolk platelets are increased in number. Stage 5 oocyte is about 1000-1200 um in diameter with dark brown animal pole and light brown vegetal pole. Most pigment granules exist at the animal pole. Pinocytotic vesicles are still present in this stage. Nuclear membrane is highly sacculated. Stage 6 oocyte is 1200-1300 um in diameter with white to cream colored vegetal pole. Pinocytotic vesicles are not observed. Large yolk platelets are found at the vegetal pole whereas smaller platelets are exist at the animal pole.

Ultrastructurally the follicle cells of the small previtellogenic oocytes have the appearance of fibroblasts and contain very few organelles. In the hot season, the follicle layer becomes hypertrophied, and the appearance of the glandular cells.

Recruitment of previtellogenic oocytes to vitellogenic growth commenced in the hot season (March) through the wet season. It was found that the mature stage 6 oocytes were observed during May to December. The presence of stage 6 oocytes during May to August was correlated with the large amount of spermatozoa in the testis in male frogs. The amplexing occurred two times in the months of high rainfall (May and August) associated with high soil moisture together with surplus water forming temporary pond for breeding site. Thus, the period from the late May to August was referred as breeding time.

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APPENDIX

Harris' Hematoxylin and Eosin Stain (Luna, 1968)

Solutions

1. Harris' hematoxylin

Hematoxylin crystals	5.0 g
Absolute alcohol	50.0 ml
Ammonium alum	100.0 g
Distilled water	1000.0 ml
Mercuric oxide (red)	2.5 g

Dissolve hematoxylin in alcohol, alum in distilled water by heat. Remove from heat and mix the two solutions. Bring to a boil as rapididly as possible. (Limit the heat to less than 1 minute and stir often). Remove from heat and add mercuric oxide slowly. Reheat to a simmer until it becomes dark purple, remove from heat immediately and plunge the vessel into a basin of cold water until cool. The stain is ready for use as soon as it cools. Addition of 2-4 ml of glacial acetic acid per 100 ml of solution increase the precision of the nuclear stain. Filter before use.

2. Acid alcohol

Alcohol, 70 %	1000.0 ml
Hydrochloric acid , concentrated	10.0 ml

3. Ammonia water

Ammonium hydroxide, 28%	2-3 ml
Tap water	1000.0 ml

4. Eosin - phloxine solution

Stock eosin

Eosin Y, water soluble	1.0 g
Distilled water	100.0 ml

Stock phloxine

Phloxine B	1.0 g
Distilled water	100.0 ml

Working solution

Stock eosin	100.0 ml
Stock phloxine	10.0 ml

Alcohol, 95%	780.0	ml
Glacial acetic acid	4.0	ml

Make up working solution as needed. Working solution should be changed at least once a week.

5. Lugol' s solution

Iodine	1.0	g
Potassium iodide	2.0	g
Distilled water	100.0	ml

6. 5% Sodium thiosulfate

Sodium thiosulfate	5.0	g
Distilled water	100.0	ml

Staining procedure

1. Deparafinize and hydrate to water.
2. If section are Bouin – Hollande sublimate fixed, remove mercuric precipitated in Lugol's solution 10 min and clear in sodium thiosulfate solution 5 min. Wash in tap water and rinse in distilled water.
3. Harris's hematoxylin for 10 – 15 min.
4. Rinse in tap water.
5. Differentiate in acid alcohol, two to three quick dips. Check the differentiation with a microscope. Nucleus should be distinct and the background very light or colorless.
6. Wash in tap water
7. Dip in ammonia water until sections are bright blue.
8. Wash in running tap water 5 – 10 min
9. Stain in eosin 1 – 2 min
10. Dehydrate in 95% alcohol, two changes, 2 min each.
11. Absolute alcohol, two changes, 5 min each.
12. Clear in xylene, three changes, 5 min each.
13. Mount with Permount.

Results :

Nuclei - blue

Cytoplasm - various shades of pink

Electron Microscopic Study

Solutions

1. 0.1M Cacodylate buffer

Solution A (0.2 m)

Sodium cacodylate	21.4	g
Distilled water	1000.0	ml

Solution B

Hydrochloric acid	10.0	ml
Distilled water	603.0	ml

Mix 50 ml solution A and 2.7 ml solution B and add 50 ml distilled water

2. 3% Glutaraldehyde fixative in 0.1 M cacodylate buffer

Glutaraldehyde, 25%	12.0	ml
0.2 M Cacodylate buffer	50.0	ml

Adjust pH to 7.3 with 1 M HCl . Dilute to 50 ml with distilled water. Store in refrigerator.

3. 1% Osmium tetroxide in 0.1 M cacodylate buffer

Stock solution of 2% osmium tetroxide

Breaking ampules containing 1 g of osmium tetroxide and dropping into a clean glass brown bottle to which is added 50 ml distilled water. Allow at least 24 hours for the osmium tetroxide to disolve. Store in refrigerator.

Working solution

2% Osmium tetroxide stock solution	10.0	ml
0.2 M Cacodylate buffer	10.0	ml

Store in refrigulator.

4. 2% Uranyl acetate aqueous solution

Uranyl acetate	2.0	ml
Distilled water	100.0	ml

Store in refrigulator, and filtrate before use.

5. Poly/Bed 812 - Araldite mixture

Poly/Bed 812	10.0	ml
Araldite	10.0	ml
DDSA	24.0	ml

DMP-30	0.9	ml

6. Lead citrate

Lead nitrate	1.33	g
Sodium citrate	1.76	g
Distilled water		

Shake well, add 8 ml 1 N sodium hydroxide, and distilled water to make up total volume to 50 ml.

7. 4% Uranyl acetate aqueous solution

Uranyl acetate	4.0	g
Distilled water	100.0	ml

CURRICULUM VITAE

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EDUCATION : Year	INSTITUTION	DEGREE/DIPLOMA
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